ASSESSMENT OF VIRULENCE PROPERTIES AND CHARACTERIZATION OF ENVIRONMENTAL *AEROMONAS* SPP. BY BIOCHEMICAL, SEROLOGICAL AND MOLECULAR TECHNIQUES

A DISSERTATION SUBMITTED TO THE UNIVERSITY OF DHAKA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY
FACULTY OF BIOLOGICAL SCIENCE
UNIVERSITY OF DHAKA
BANGLADESH

SUBMITTED BY:
MUHAMMED ZEAUR RAHIM
REG. NO. 14/96-97

Dedicated to my family

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ABSTRACT

Aeromonas spp. were isolated from duckweed (DW), water and gills and intestinal contents of fish sampled from wastewater and non-wastewater areas of a fish farm. Among the three species of *Aeromonas*, the incidence of *A. sobria* (56.6%) was greater than that of *A. hydrophila* (28.0%) and *A. caviae* (15.8%). No significant difference was observed between wastewater and non-wastewater areas in terms of density of *Aeromonas* in DW (P=0.053), water (P=0.384) and gills (P=0.402) and intestinal contents (P=0.312) of fish samples. The density of *Aeromonas* in gills significantly correlated (P<0.001) with that of intestinal contents of fish in both the areas. The density of *Aeromonas* in gills and intestinal contents of fish also correlated with pH (P< 0.001) and conductivity (P<0.001) only in the non-wastewater area and with temperature in both wastewater (P<0.01) and non-wastewater areas (P<0.001). *Aeromonas* density in water correlated with conductivity (P<0.001) only in the wastewater area. This study concludes that DW cultivated for wastewater treatment can safely be used as fish-feed.

Aeromonas hydrophila phages were isolated from water and sediment samples of both sewage-contaminated and sewage-free ponds. Density of *Aeromonas* phages ranged between $4.0x10^1$ and $2.0x10^4$ pfu/ml or g in water and sediment samples. This study concludes that *Aeromonas* phages are distributed in both sewage- contaminated and uncontaminated ponds.

Cytotoxic enterotoxin (*act*) gene was detected in 32 of 69 environmental isolates of *Aeromonas* spp. Gene-positive isolates significantly differed from gene-negative ones with respect to enterotoxicity in suckling mice assay (P=0.009) and hemolytic activity (P=0.005). However, all *act* gene-positive and gene-negative isolates of *Aeromonas* were uniformly sensitive to chloramphenicol, ciprofloxacin and furazolidone.

The highest titer of cytotoxic enterotoxin was produced in brain heart infusion broth (BHIB) compared to trypticase soy broth with yeast extract, casamino acid yeast extract and Richardson's medium. Production of cytotoxin was enhanced when BHIB was supplemented with calcium (1 mM), magnesium (10 mM), iron (200 μg) and sodium chloride (0.5%).

Pilus was detected in 13 isolates and O:34 antigen in 11 isolates of *Aeromonas* spp. harboring *act* gene. Pili and O:34 antigen were detected more among *A. sobria* than in *A. hydrophila* isolates. However, no relationship could be established between *act* gene and production of pilus and O:34 antigen in *Aeromonas* spp.

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List of Abbreviations

A	: *Aeromonas*
sp.	: species
app.	: specieses
%	: percentage
c.f.u	: colony forming unit
e.g.	: as for example
et al.	: and others/ et alli
fig.	: figure
g	: gram
h	: hour
J	: journal
LB	: Luria Bartani
mM	: mili mole
µM	: micro mole
mS/m	: millisiemens/m
pH	: negative logarithm of hydrogen ion concentration

CHAPTER - 1

INTRODUCTION AND REVIEW OF LITERATURE

1. INTRODUCTION AND REVIEW OF LITERATURE

1.1. INTRODUCTION

Diarrhea is one of the major causes of illness and death among children in under-developed and developing countries of the world (Snyder and Merson 1982). It is estimated that 1.3 thousand million episodes and 4 million deaths occur each year among children, aged less than five years, due to diarrhea. Globally, children experience an average of 3.3 episodes of diarrhea each year but in some parts of the world it exceeds 9 episodes each year (Bern *et al.* 1992). In endemic areas, 80% of deaths due to diarrhea occur in the first two years of life (WHO 1990).

Diarrhea, a water-borne disease, is transmitted to susceptible host through the fecal-oral route. Due to lack of facilities for safe disposal of excreta, groundwater and surface water become contaminated with enteric pathogens. As a result of using contaminated waters for bathing, swimming and washing of utensils, enteric pathogens enter into the gut to initiate infection when the pathogen is highly virulent. The avirulent or moderately virulent pathogens cannot cause enteric infection until they acquire virulence through repeated passage in the gut of community dwellers as a result of constantly consuming polluted waters. This phenomenon was proved when stains of *Aeromonas* spp., acquired virulence through repeated passage in the gut of laboratory animals (Singh and Sanyal 1996, Krzyminska *et al.* 2001). Thus, being passaged in the community, virulent strains of *Aeromonas* are released in the environment through leakage of sewage. These virulent strains of *Aeromonas*, which are prevalent in the community, can cause community-wide outbreak of diarrhea.

A few years ago, causative agents of diarrhea could be identified in the feces of only 25% patients with acute diarrhea. However, with the advancement of science and introduction of innovative techniques, it is now possible to identify more than 80% of causative agents (WHO 1990). The etiological agents of diarrhea are numerous and some of them are known for decades. Most of the agents are distributed world-wide but some of them are endemic in certain geographical regions due to unique behavior of pathogens, environmental condition, behavioral patterns of the host etc. New pathogens are continuously being added to the list of diarrhea causing agents. Newly identified agents are *Providentia alcalifaciens* (Rahman *et al.* 2002), *Aeromonas* spp. (Merino *et al.* 1995) and *Escherichia coli* (Albert *et al.* 1995).

Aeromonas spp. are abundant in natural water bodies (Gibotti *et al.* 2000). They can be isolated from fresh water (Pasetto *et al.* 1998), drinking water supplies (Legnani *et al.* 1998) and bottled water (Warbutton *et al.* 1994) as well. Density of *Aeromonas* spp. fluctuates with the variation in physicochemical parameters of water in an aquatic ecosystem (Fliermans *et al.* 1977). *Aeromonas* spp. show population dynamics in natural water (Pettibone 1998) and in sewage treatment pond (Monfort and Baleux 1990). Strains of *Aeromonas* spp. have also been isolated from polluted waters (Araujo *et al.* 1991). From the contaminated water, soft tissue infection in human beings caused by this organism has been reported (Joseph *et al.* 1979, Sacho *et al.* 1990). Moreover, it has been isolated from chlorinated and unchlorinated domestic water supplies (Burke *et al.* 1984a, 1984b). Fluctuation of aeromonad density in unchlorinated domestic water correlates with clinical isolates from cases of diarrhea (Burke *et al.* 1984a). Besides, *Aeromonas* spp. are important fish pathogen (Rahim *et*

al. 1984, Chattopadhyay *et al.* 1991, McGray *et al.* 1991) and can adversely affect fish industry severely. *Aeromonas*-infested water induces mortality of fingerlings in the hatchery (Ortega *et al.* 1996).

In the early 1960s, Lautrop (1961) isolated *A. hydrophila* from feces of diarrheal patients and suspected its association as a causative agent. Subsequently, Rosner (1964) isolated this bacterium from feces of a diarrheal patient with abdominal pain, fever and bloody stools. Rosner (1964), for the first time, proved association of *A. hydrophila* with diarrhea by agglutination of the isolated bacterial strain with the patient's serum. Importance of *Aeromonas* spp. as a diarrhea causing agent became more alarming when isolated from 5.2% of patients from India (Bhat *et al.* 1974) and 8.6% of patients in Bangladesh (Aziz *et al.* 1986) during cholera-like epidemics. Isolation rate of *Aeromonas* spp. from diarrheal stool differs in different geographical settings (Gracey *et al.* 1986). Among different age groups, the incidence of *Aeromonas* is notably higher among children (Burke *et al.* 1984a, Teka *et al.* 1999).

Published literature on *Aeromonas* spp. has recognized this bacterium as a versatile pathogen causing different types of infection in humans and in domestic and aquatic animals. Different fish species are infected with ulcerated disease every year in Bangladesh. However, research related to ecology of *Aeromonas* spp. in fish farms has not been addressed. Therefore, study related to correlation of density of *Aeromonas* with physicochemical parameters of water will be of interest. Furthermore, characterization of isolates of *Aeromonas* spp. by biochemical, serological and molecular techniques can also be explored.

1.2. REVIEW OF LITERATURE

1.2.1. Taxonomy: The bacterium *Aeromonas* was first isolated by Zimmerman in 1890 in early nineteen century from tap water and he named it as *Bacillus punctatus*. One year later, Sanarelli isolated a similar type of bacterium from frog infected with a disease called "red leg disease" (Sanarelli 1891). Sanarelli named this bacterium as *Bacillus hydrophilus fuscus*. Finally, Kluyver and Van Niel (1936) proposed the genus *Aeromonas* to describe the bacteria isolated by Zimmerman (1890) and Sanarelli (1891). Finally, the genus *Aeromonas* was included in the 7th edition of Bergey's Manual (Breed *et al.* 1957). Subsequently, Veron (1966) proposed to include *Aeromonas* in the family of *Vibrionaceae.* In the 8th edition of Bergey's Manual (Buchanan and Gibbons 1974), the original definition of the genus *Aeromonas* was amended with the following salient properties: Gram-negative strait rod with polar flagella (generally monotrichous) or non- motile; facultative aerobes; fermenting cabohydrates with the formation of acid with and without gas; cytochrome-oxidase positive; reducing nitrates to nitrites; insensitive to the vibriostatic compound (2,4-diamino-6, 7-diisopropilepteridine, also called 0/129); guanine-cytosine content of the DNA 57 to 63
mol %.

The genus *Aeromonas* includes two separate groups: one is psychrophilic and non-motile, while the other is mesophilic and motile. *A. salmonicida* is included within the psychrophilic group and is mostly associated with infection of fish (von Graevenitz 1985). The mesophilic group is divided into three species: *A. hydrophila*,

A. sobria and *A. caviae* (Popoff 1984). Species of the mecophilic group is associated with infection of humans (Janda *et al.* 1998) and other animals.

Aeromonads have been placed under the family *Vibrionaceae* based on phenotypic characteristics. But molecular biological evidence, including nucleotide sequence of 16S and 5S rRNA and rRNA-DNA hybridization data, suggest that aeromonads are different from the members of *Enterobacteriaceae* and *Vibrionaceae.* All these evidence suggest that the species of the genus *Aeromonas* represent a distinct family *Aeromonadaceae* fam. nov (Colwell *et al.* 1986).

Analysis based on the electrophoretic variation of different cellular enzymes along with DNA-DNA hybridization data of different strains of *Aeromonas* indicates that the genus is composed of several species designated as hybridization group (HGs) (Janda *et al.* 1991). Published reports recognize 12 HGs (Janda *et al.* 1991). Since 1987, a number of new *Aeromonas* spp. have been proposed and a total of 14 *Aeromonas* spp. along with the HGs have been reported (Hickman-Brenner 1987, Hickman-Brenner 1988, Carnahan *et al.* 1991a, Carnahan *et al.* 1991b, Janda 1991, Martinez-Marcia *et al.* 1992, Esteve et *al.* 1995, Ali *et al.* 1996). These species are shown in Table-1.1 along with their potential role in human infection and source of isolation. Table-1.1 also shows two biotypes of *A. veronii* (*A. veronii* biotype sobria and *A. veronii* biotype veronii).

1.2.2. Serotyping of *Aeromonas* spp.: Serotyping is the best method of differentiating biochemically similar strains having a different antigenic structure. Moreover, the serotyping scheme can be precisely used as epidemiological markers.

During epidemic, isolation of an etiological agent(s) of a particular serotype may provide sufficient clue to trace the probable route of transmission and to formulate effective and appropriate strategies for intervention.

Preliminary characterization of somatic and flagellar antigen of *Aeromonas* was conducted at the Center for Disease Control (CDC), USA. (Ewing *et al.*1961). Unfortunately, these representative reference strains were not available at CDC to pursue further typing of *Aeromons* strains. Two decades later, Leblance *et al.* (1981) proposed another scheme for serotyping mesophilic fish isolates of *Aeromonas*. They divided 195 motile *Aeromonas* into 12 O-antigenic groups without designating antigenic symbol. This serotyping scheme was considered incomplete as the studied strains had common source of isolation.

Table-1.1. Species of *Aeromonas*, their probable pathogenic role and source of isolation

Aeromonads associated as		**Non-pathogen**
Major pathogens	Minor pathogens	Environmental species
A. hydrophila (HG 1)	*A. veronii* bt veronii (HG 10)	*A. salmonicida* (HG 3)
A. caviae (HG 4)	*A. jandaei* (HG 9)	*A. sobria* (HG 7)
A. veronii bt sobria (HG 8)	*A. schubertii* (HG 12)	*A. media* (HG 5)
		A. eucrenophila (HG 6)
		A. trota
		A. allosaccharophila
		A. encheleia (HG 11)
		A. bestiarum (HG 2)
		A. popoffii

Considering the limitations of the previously proposed serotyping scheme of *Aeromonas*, Sakazaki and Shimada (1984) put forward another new scheme for serotyping mesophilic motile *Aeromonas* spp. collected from diverse sources and different geographical locations. They defined 44 O-serogroups and also pointed out that some O-antigens of *Aeromonas* were identical or closely related to some other serogroups of *Vibrio fluvialis* and *Plesiomonas shigelloides*.

The Division of Enteric Pathogen of Central Public Health Laboratory (Cheasty *et al.* 1988), Colindle, U.K., raised antiserum corresponding to O-antigenic serogrouping of Sakazaki and Shimada (1984). This serotyping scheme was followed to type 864 mesophilic motile *Aeromonas* isolated from 775 samples of human feces, 36 clinical specimens other than feces and 53 non-human samples of the United Kingdom. Using the scheme of Sakazaki and Shimada (1984), 40% *Aeromonas* strains of U.K. were sero-typed. But the typing ability rate differed in different species of *Aeromonas*. In this scheme, 10 or more O-serogroups of *Aeromonas* spp. were detected. Cheasty *et al.* (1988) realized further expansion of the serotyping scheme for improving typing ability of strains of *Aeromonas* spp.

Misra *et al.* (1989) serotyped 118 mesophilic strains isolated from diarrheal stools and environmental samples of Calcutta, India using the serotyping scheme of Sakazaki and Shimada (1984). They were able to type *Aeromonas* strains into 23 different serogroups. The serogroups were O:34, O:16 and O:11 in order of predominance. But none of the above-mentioned workers could comment on epidemiological and pathogenic implications of a particular stereotype.

Aeromonas spp. isolated from clinical presentations of human disease (septicemia, meningitis, peritonitis and wound infection) (Kokka and Janda 1990, Khashe *et al.* 1996) and epizootic outbreaks of gold fish septicemia (Merino *et al.* 1988) were serotyped using the Sakazaki and Shimada (1984) scheme. Two predominant serogroups, such as O:11 and O:34 were detected which were established as pathogenic serotypes of *Aeromonas* species.

Considering the limitation of the Sakazaki and Shimada (1984) scheme for serotyping *Aeromonas* spp., Thomas *et al.* (1990) proposed an extended serogrpouing scheme for typing motile mesophilic *Aeromonas* spp. Subsequently, Shimada and Kosako (1991) compared the existing two O-serogrouping schemes of *Aeromonas* and found a good agreement between them.

Further studies on biochemical characterization and serological properties of the genus *Aeromonas* indicated that one genospecies includes more than one stereotype and the reference strains for each hybridization group are not serologically representative of a particular genospecies. Serotype O:11, O:16 and O:34 were the predominant serotypes among clinical isolates (Janda *et al.* 1996).

Merino *et al.* (1994) reported complement-mediated killing of *Aeromonas* strains of serotype O:34 at 37°C (serum-sensitive). These strains could resist (serum-resistant) complement-mediated killing when grown at 20°C. It was investigated that at low temperature, strains of *Aeromonas* produce smooth polysaccharide, which helps bacteria to resist complement-mediated killing. But when cultured at elevated temperature (37°C), the same bacteria produced rough polysaccharide, which enhances complement-mediated killing of *Aeromonas* spp.

Strains of *A. hydrophila* belonging to serotype O:11 and O:34 can produce capsule (Merino *et al.* 1995). Moreover, some strains of *A. trota* cross-react with antiserum of *Vibrio cholerae* O139. These strains of *A. trota* are pathogenic (Albert *et al.* 1995). These cross-reacting *A. trota* strains possessed slightly different type of O-specific polysaccharide which were responsible for cross-reacting with antiserum of *V. cholerae* O139 Bengal (Knirel *et al.* 1996).

1.2.3. Distribution of aeromonads in aquatic environment

Aeromonas spp. are ubiquitously distributed in the aquatic environment. Strains of this genus are abundant in surface water (Fliermans *et al.* 1977, Hazen *et al.* 1978, Kersters *et al.* 1995), ground water (Kersters *et al.* 1995), brackish water (Kaper *et al.* 1981), sewage and wastewater (Boussaid *et al.* 1991), chlorinated and unchlorinated domestic water supplies (Burke *et al.* 1984a, 1984b), wastewater (Hassani *et al.* 1992), stabilization pond (Boussaid *et al.* 1991), wastewater purification plants (Stecchinis and Domenis 1994) etc. Even, *Aeromonas* spp. have been isolated as contaminant in bottled water samples of Saudi Arabia (Slade *et al.* 1986), Spain (Gonzalez *et al.* 1987) and Canada (Warburton *et al.*1992). Isolation of *Aeromonas* spp. from diverse aquatic ecosystem indicates unique adaptive capability of this organism (Mateos *et al.* 1993) in diverse ecological niches. It is suggested that human beings acquire intestinal infection mostly due to consumption of *Aeromonas*-infested raw and processed drinking water (Van der Kooij 1988). It is further assumed that under certain circumstances, current technology for the production of drinking water may provide environmental conditions favoring growth of aeromonads

(Schubert 1991, Kersters *et al.* 1995). In fresh and nutrient-poor water, the survival potential of *Aeromonas* also varies (Kersters *et al.* 1996).

Different physicochemical parameters of water may affect the growth and distribution of *Aeromonas* in the aquatic environment. Two decades from nineteen seventies, effect of physicochemical parameters of water on *Aeromonas* in the aquatic ecosystem was extensively studied by different investigators. These studies mostly focused on the effect of dissolved oxygen (Seidler *et al.* 1980), temperature (Hazen *et al.* 1978), pH (Hazen *et al.* 1978) and conductivity (Burke *et al.* 1984a) etc.

Relationship of dissolved oxygen with density of *Aeromonas* in an aquatic ecosystem was first raised by Seidler *et al.* (1980). An inverse relationship between dissolved oxygen and density of *Aeromonas* was noted in a study conducted in the Anacostia river (Seidler *et al.* 1980). Study of Kaper *et al.* (1981) in the Chesapeake Bay confirmed the observations of Seidler *et al.* (1980). Further studies are needed outside the USA to confirm the observations of thse groups.

Aeromonas can grow at a wide range of pH (5.0 to 9.0) but unable to grow at a pH lower than 4.0 and higher than 10 (Hazen *et al.* 1978). Besides, the optimum growth temperature of *Aeromonas* was close to 45°C but most of the strains of *Aeromonas* can grow at 35°C (Rouf and Rigney 1971). Temperature is one of the important factors that affects the growth and distribution of *Aeromonas* spp. in the aquatic environment (Kaper *et al.* 1981). Cavari *et al.* (1981) demonstrated elevated metabolic activity of *A. hydrophila* at 25°C compared to 4°C in a laboratory-based experiment and ultimately explained the possible correlation of increasing the density of *Aeromonas* due to gradual rise of water temperature in the Anacostia river of USA

(Seidler *et al.* 1980). Positive correlation of density of *Aeromonas* with water temperature was reported by several investigators (Hazen *et al.* 1978, Hazen and Fliermans 1979, Rippey and Cabelli 1979, Seidler *et al.* 1980, Kaper *et al.* 1981). However, an inverse relationship of density of *Aeromonas* with water temperature was reported by Burke *et al.* (1984a) and Boussaid *et al.* (1991). Further study is needed to explain the discrepancy between the findings of different investigators of different geographical locations.

Conductivity of water in the aquatic environment is influenced by different metallic ions. Some of these metallic ions are used as enzymatic co-factors for growth, metabolic activity and survival of bacteria in the aquatic ecosystem. Thus it is assumed that conductivity might play an important role in the survival of *Aeromonas* in the aquatic ecosystem. Burke *et al.* (1984a) reported significant correlation of density of *Aeromonas* with conductivity of water.

1.2.4. Human infection

Aeromonas enters into the history of human infection as a result of its isolation from different autopsy materials of a 40-year-old Jamaican lady who died of an acute disease called "Fulminating metastatic myositis." (Hill *et al.* 1954). Recently, Caselitz (1996) has described the detailed history of the introduction of *Aeromonas* in Medical Microbiology. Now a days, a huge number of publications are available in biomedical journals indicating different types of *Aeromonas* spp. associated human infections.

1.2.4.1. Intestinal infections: *Aeromonas* spp. associated diarrhea was first reported in 1961 in Colambia during an outbreak of diarrhea in a nursery for neonates

(Martinez-Silva *et al.* 1961). In the same year, Lautrop (1961) isolated this bacterium from feces of diarrheal patients and suspected its pathological role in association with diarrhea. Subsequently, Rosner (1964), for the first time, proved the association of this bacterium with diarrhea by agglutinating isolates with patient's serum. In early 1970s, *Aeromonas* attracted attention of medical scientists when it was isolated from 5.2% of diarrheal patients from India (Chatterjee and Noegy1972, Bhat *et al.* 1974). However, evidence of *Aeromonas* in association with diarrhea came until Gracey *et al.* (1982) reported a case-control study of *Aeromonas* associated diarhea in Australia. In this study, *Aeromonas* was isolated from 10% of cases with diarrhea but rarely from healthy controls. Results of other case-control studies conducted in the USA (Agger *et al.* 1985, Holmberg *et al.*1986) also supported *Aeromonas* as diarrheal pathogen.

Diarrhea associated with *Aeromonas* spp. prevails throughout the world. But reportes are avialable from some countries, such as Tropical Queensland, Australia, (Ashdown and Koehler 1993), West Australia, Perth (Gracey *et al.* 1982), Bangladesh (Hossain *et al.* 1991, Teka *et al.* 1999), Brazil (Guerrant *et al.* 1975), Canada (Gurwith and Williams 1977), England (Millership *et al.* 1983, Wilcox *et al.* 1992, Shread *et al.* 1981), Ethiopia (Wadstrom *et al* 1976a), India (Chatterje and Neogy 1972, Ram *et al.* 1991), Israel (Gluskin *et al.* 1992), Nigeria (Ogunsanya *et al.*1994), Peru (Pazzaglia *et al.* 1991), Sweden, (Svenungsson *et al.* 2000), Thailand (Pitarangsi *et al.* 1982) and the USA (Agger *et al.* 1985). Isolation rates of *Aeromonas* spp. from stool specimens from different geographical areas are shown in Tables-1.2 and 1.3.

1.2.4.1.1. Types of diarrhea: Clinical manifestations of *Aeromonas* associated diarrhea can be categorized into four types: a) acute watery diarrhea often with vomiting (Burke *et al.* 1983, Gracey *et al.* 1982), b) acute diarrhea characterized by blood and mucus in the stool (Rahman and Willoughby 1980, Marsik and Werlin 1984), c) diarrhea presenting for >10 days (Baman 1980, Palfreeman *et al.* 1983), d) rice watery stool (Champsaur *et al.* 1982, Gelbert *et al.* 1985). *Aeromonas* spp. can also cause travellers' diarrhea (Gracey *et al.* 1984).

1.2.4.1.2. Travelers' diarrhea: Diarrheal episodes during and after traveling parts of the world are called travelers' diarrhea. Every year 20 to 56% diarrheal episodes occur among 12-20 million travelers from the industrialized countries to different parts of the world (DuPont *et al.* 1987, Black 1990). Enterotoxigenic *Escherichia coli* (ETEC) was the most common bacterial agent along with other bacterial, viral and parasitic agents associated with travelers' diarrhea (Black 1990). Travelers' diarrhea shows seasonal variation of different etiological agents (Mattila *et al.* 1993).

Aeromonas was suggested as one of the potential agents associated with travelers' diarrhea. Echeverria and co-workers (1981) initially suspected the etiological role of *Aeromonas* in travelers' diarrhea. They found diarrhea in American Peace Corps Volunteers (PCVs) in Thailand. In their study, *Aeromonas* was isolated from 30% and 8.5% of stools respectively from diarrheal patients and healthy American PCVs. Subsequently, isolation of *Aeromonas* from travelers' diarrhea has been reported occasionally (Rahman and Willoughby 1980, Speelman 1983) without emphasizing the role of *Aeromonas* as an etiological agent of travelers' diarrhea. Subsequently, Gracey *et al.* (1984) first pointed out that *Aeromonas* might be an etiological agent of

travelers' diarrhea. They reported eight cases of travelers' diarrhea where *A. hydrophila* was isolated in pure culture from stool. In Finland, *A. veronii* biotype sobria (hybridization group 8/10) and *A. caviae* (HG 4) were isolated most commonly from *Aeromonas*-associated travelers' diarrheal episodes (Hanninen *et al.* 1995). *Aeromonas*-associated travelers' diarrhea symptom also varies among patients (Yamada *et al.* 1997).

1.2.4.2. *Aeromonas*-associated other human diseases: Other than diarrhea, *Aeromonas* spp. are associated with a number of important human infections. These includes; hemolytic-uremic syndrome (Bogdanovic *et al.* 1991, Robson *et al.* 1992, Fang *et al.* 1999), ocular infection (Sire *et al.* 1990, Carta *et al.* 1994, Agulla *et al.* 1997, Lee *et al.* 1997), wound infection (Newton and Kennedy 1993, Davis *et al.* 1993, Kienzle *et al.* 2000, Itoh *et al.* 1999), peritonitis (Munoz *et al.* 1994, Ruiz de Gonzaler *et al.* 1994, Elcuaz *et al.* 1995), meningitis (Parras *et al.*1993, Sarasqueta *et al.* 1998, Lin and Cheng 1998), respiratory tract infection (Spencker *et al.*1991, Hur *et al.*1995) and septicemia (Gupta *et al.* 1996, Chang *et al.* 1997, Steinfeld *et al.* 1998, Hoshina *et al.* 1999, Tabata *et al.* 1999).

Table-1.2. Isolation rate of *Aeromonas* spp. from diarrheal stools in some countries

Country	Rate of isolation	Reference
Australia (Tropical Queensland)	4.9% in all age	Ashdown and Koehler 1993
Australia (West, Perth)	>10% children	Gracey *et al.* 1982
Bangladesh	6.5% children	Teka *et al.* 1999
Bangladesh	8.5% children	Hossain *et al.* 1991
Brazil	0	Guerrant *et al.* 1975
Canada	<0.3% all ages	Gurwith and Williams 1977
England	10.6% all ages	Millership *et al.* 1983
England	8.5% all ages	Shread *et al.* 1981
England	2.5% all ages	Wilcox *et al.* 1992
Ethiopia	3.2% children	Wadstrom *et al* 1976a
India	4.9% all ages	Chatterjee and Neogy 1972
India	2.1% all ages	Ram *et al.* 1991
Israel	4% children	Gluskin *et al.* 1992
Nigeria	1.4% children	Ogunsanya *et al.* 1994
Peru	52.4% all ages	Pazzaglia *et al.* 1991
Sweden	2% adults	Svenungsson *et al.* 2000
Thailand	16.2% adults	Pitarangsi *et al.* 1982
United States of America	1.1% children	Agger *et al.* 1985

Individuals of all ages are affected with *Aeromonas*-associated gastritis. However, children are mostly affected (Gracey *et al.* 1982, Ogunsanya *et al.* 1994, Teka *et al.* 1999).

Table-1.3. Isolation rate of *Aeromonas* spp. from non-diarrheal stools in some countries

Country	Rate of isolation	Reference
Australia	0.7% children	Burke *et al.* 1983
Bangladesh	1.5% children	Hossain *et al.* 1991
England	1.25% all ages	Shread *et al.* 1981
England	3.3% all ages	Millership *et al.* 1983
France	0 adults	Catsaras and Buttiaux 1965
France	0.7% children	Catsaras and Buttiaux 1965
India	0 all ages	Ram *et al.* 1991
India	0	Deodhar *et al.* 1991
Thailand	10% adults	Pitarangsi *et al.* 1982
United States of America	3.2% all ages	von Graevenitz and Zinterhofer 1970
United States of America	0	Agger *et al.* 1985

1.2.5. Pathogenic mechanisms:

Adhesion is the first step in pathogenesis of *Aeromonas* like other bacterial pathogens, such as *Vibrio cholerae* (Finkelstein *et al.* 1983) and *V. parahaemolyticus* (Oishi *et al.* 1979). Adhesion of *Aeromonas* spp. is mediated by hemagglutinin, pilus, adhesins for binding extracellular matrix etc.

1.2.5.1. Hemagglutinin: Hemagglutination is widely used as an indicator of adhesion mediated by hemagglutinin(s). *Aeromonas* spp. possess soluble (Stewart *et al.* 1986) and cell-associated hemagglutinins (HA) like that of *V. cholerae* (Finkelstein *et al.*

1983) and *V. parahaemolyticus* (Oishi *et al.* 1979). Hemagglutinins are classified in two types: soluble hemagglutinin and cell-associated hemagglutinin.

1.2.5.2. Soluble hemagglutinin (SHA): HA which is detected in the cell-free culture supernatant is called SHA. SHA of *Aeromonas* shares many properties with those of *V. cholerae* in terms of molecular weight, sugar inhibition, sensitivity to a particular temperature, Ca^{++}, or reducing agents etc. SHA of *Aeromonas* and *Vibrio* also differ from each other. In contrast to *V. cholerae*, SHA of *Aeromonas* is defficient in proteolytic activity (Stewart *et al.* 1986). It has been detected in three species of *Aeromonas* (*A. sobria*, *A. hydrophila* and *A. caviae*). Even the hemolytic strains of *Aeromonas* spp. possess SHA (Burke *et al.* 1984c).

1.2.5.3. Cell-associated hemagglutinin (CAHA): HA which is not released into the cell-free culture supernatant is called CAHA. Different patterns of CAHA of *Aeromonas* spp. with respect to different sugar and source of erythrocytes have been reported (Atkinson and Trust 1980, Adams *et al.* 1983, Burke *et al.* 1984c, Crichton and Walker 1985). Hemagglutination pattern also varies with the source of isolation of *Aeromonas*. Fucose-resistant hemagglutination of erythrocytes from group O blood of human was found in 60% of *Aeromonas* isolated from non-diarrheal stool, 86% of isolates from diarrheal stool and only 20% of isolates from the environment (Burke *et al.* 1984). *Aeromonas* isolates from diarrhea possess mannose-sensitive hemagglutinin in contrast to mannose-resistant diarrheagenic isolates of *Escherichia coli* (Satterwhite *et al.* 1978). *Aeomonas* possess pili but hemagglutinating ability of different *Aeromonas* pili is different (Atkinson and Trust 1980). Moreover, the

pathogenic potential of *Aeromonas* does not correlate with a particular hemagglutination pattern of an isolate.

1.2.5.4. Adhesion of mammalian cell-line: Other than hemagglutination, attempt was made to correlate the pathogenic potential of *Aeromonas* isolates with adhesion of mammalian cell-lines. Adhesion of HEp-2 sell-line was initially used as a model to study pathogenicity of *E. coli* (Tavendale and Old 1985). Subsequently, Carrello *et al.* (1988) found that this model was useful to study intestinal infection using *Aeromonas* spp. isolated from clinical and environmental sources. Neves *et al.* (1994a) reported aggregative types of adhesion of *Aeromonas* to HEp-2 cell-line. Contrast to Neves *et al.* (1994a), Thornley *et al.* (1994) reported aggregative type of adhesion by strains of *A. caviae*, but they did not examined their isolates for pili. However, non-piliated clinical and environmental isolates of *A. hydrophila* also adhered to HEp-2 cell-line (Bartkova and Ciznar 1994). Environmental isolates of *A. hydrophila* showed diffused type of adhesion but 50% of *A. hydrophila* isolates showed localized type of adhesion. Adhesion of non-piliated *A. hydrophila* isolates to HEp-2 cell-line correlated with hydrophobicity of isolates (Bartkova and Ciznar 1994). However, adhesion pattern of *Aeromonas* isolates varies with cell lines, culture media and geographical origin of the isolates (Neves 1994b).

1.2.5.5. Pili of *Aeromonas* spp.: Filamentous structure, thinner and shorter than flagella, which are projected from the periphery of the bacterial cell wall, is called pili. It is also called fimbrae. This organ helps bacteria to colonize on a particular surface. Five different types of *A. hydrophila* pili (Sato *et al.* 1989, Honma and Nakasone 1990, Ho *et al.* 1990, Hokama *et al* 1990) and one type of *A. sobria* pili

(Hokama and Iwanaga 1991) have been reported from Japan. These pili have been morphologically differentiated into straight (Sato *et al.* 1989, Honma and Nakasone 1990, Ho *et al.* 1990) and flexible (Ho *et al.* 1990, Hokama *et al.* 1990, Hokama and Iwanaga 1991). Usually, the flexible type of pilus is hemagglutinating but straight ones are non-hemagglutinating. However, pilus of strain *A. sobria* ATP13 is non-hemagglutinating. It is flexible and adheres only to rabbit intestinal epithelium (Iwanaga and Hokama 1992). Of these 6 types of pili, *A. sobria* Ael pilus and *A. hydrophila* Ae6 W pilus are associated with colonization of the intestine.

Kirov and her co-workers reported about pili of *A. sobria* (Kirov *et al.* 1995 and 1996, Barnett *et al.* 1997) and of *A. caviae* (Kirov *et al.* 1998). These pili had a tendency to form bundle and are called bundle-forming pili (Bfp).

N-terminal amino acid sequence of the pili of *A. veronii* bv sobria (Barnett *et al.* 1997, Kirov *et al.* 1996) and *A. caviae* (Kirov *et al.* 1998) showed homology with the published amino acid sequence at the conserved region of the pili of *A. sobria* Ae24 (Hokama and Iwanaga 1992), *A. sobria* ATP13 (Iwanaga and Hokama 1992), *A. sobria* Ael (Hokama and Iwanaga 1991), *A. hydrophila* Ae6 (Hokama *et al.* 1990), *A. trota* 1220 (Nakasone *et al.* 1996). All these pili also showed homology with conserved region of classical type IV pili of *Pseudomonas aeruginosa* PAK, *Neisseria gonorrhoeae* MS11 and *Dichelobacter nodosus* (Barnett *et al.* 1997, Kirov *et al.* 1998). Thus, the Bfp of *Aeromonas* are also called type IV pilus. Bfp are conserved in *Aeromonas* spp. (Barnett and Kirov 1999). Other than Bfp, *Aeromonas* produces another type of IV pilus called TAP. These two pili (Bfp and TAP) differ from each other at their N-terminal amino acid sequence and molecular weight.

Type IV Bfp are important for colonization (Kirov *et al.* 1999). But the role of Tap pili is less important for colonization compared to Bfp (Kirov *et al.* 2000). Bfp of *A. caviae* CA195 are highly adhesive to HEp-2 cell-line (Kirov *et al.* 1998).

1.2.5.6. Adhesins of *Aeromonas* for binding extracellular matrix: Collagens are the family of proteins located at the extracellular matrix, such as fibronectin, fibrinogen, lamelin elastin etc. (Miller *et al.* 1987, Gulberg *et al.* 1989). Cell surface of various bacterial pathogens possesses receptors for these extracellular matrix (Fitzgerald *et al.* 1984, Kastrzynska *et al.*1989). With these receptors, bacterial pathogens adhere to the extracellular cell matrix. Therefore, presence of receptors on the bacterial cell-wall corresponding to these extracellular matrix have been proposed as potential virulence factors (Speziale *et al.* 1986, Westerlund *et al.* 1989, Visai *et al.* 1990). Cell surface of *Aeromonas* possesses family of adhesins that bind with extracellular matrix components of mammalian cells, such as collagen (Ascencio *et al.* 1990) and lactoferin (Kishore *et al.* 1991, Ascencio *et al.* 1992). *Aeromonas* cell wall-associated receptors for binding extracellular matrix varies with source of isolation (Ascencio *et al.* 1991). Extracellular enzyme, such as, protease also affects binding of *Aeromonas* cells to connective tissue (Ascencio *et al.* 1991). Moreover, *Aeromonas* can selectively bind collagen type I, basement membrane and collagen type IV (Ascencio *et al.* 1990). Moreover, *Aeromonas* can selectively bind collagen type I and basement membrane of collagen type IV (Ascencio *et al.* 1990).

1.2.5.7. Invasion: Clinical presentation of dysentery-like symptom by patients with *Aeromonas* colitis (Rahman and Willoughly 1980) gives an impetus that this bacterium may be an invasive organism. But strain of *Aeromonas* isolated from

humans failed to express invasive properties in Sereny test (Johnson and Lior 1981). It is possibly due to the fact that guinea pig keratoconjunctivitis may not be a suitable model for testing invasiveness of *Aeromonas* spp. Four years later, Lawson *et al.* (1985) successfully demonstrated HEp-2 cell invasion by strains of *A. hydrophila* isolated from patients with dysentery-like symptom. These invading strains of *A. hydrophila* harbored six-megadalton plasmid. These strains could retain their invasive properties even after curing the high molecular weight plasmids. Thus, Lawson *et al.* (1985) concluded that *A. hydrophila* invasiveness is mediated by gene located in the chromosome.

1.2.6. Plasmid: It is established that virulence-associated properties of *Aeromonas*, such as cytolytic enterotoxin (β-hemolysin), invasive properties, type IV pili etc. are mediated by genes located on the chromosome (Lawson *et al.* 1985, Husslein 1988, Pepe *et al.* 1996). But other pathogenic factors, such as mini pilin (Ho *et al.* 1992), Shiga-like toxin (Haque *et al.* 1996), cell-line adhesion and hemolysin production (Hanes *et al.* 1993) are encoded by genes located on the plasmids. A recent study of Brown *et al.* (1997) indicates that very few isolates of *Aeromonas* carry plasmid(s) (16%) and plasmids are more common in environmental isolates of *A. veronii* biovar sobria than in clinical isolates of the same species

1.2.7. Enterotoxin: Initial idea of *Aerononas* enterotoxin was evolved in the late eighties when live culture of *Aeromonas* isolates from diarrheal patients could induce fluid accumulation as a result of injecting it into the rabbit ileal loop (RIL) (Sanyal *et al.* 1975). This finding provided a preliminary idea of enterotoxic potential of this bacterium. Subsequently, production of extracellular and heat labile nature of the

enterotoxin of *Aeromonas* was reported independently from Sweden (Wadstrom *et al.* 1976a) and India (Annapurna and Sanyal 1977). Enterotoxin-producing isolates of *Aeromonas* spp. have been reported from fish (Boulanger *et al.* 1977), pigs (Dobrescu 1978), brackish water (Kaper *et al.* 1981) and from divers of the Anacostia river (Seidler *et al.* 1980). Enterotoxicity of *Aeromonas* toxin was tested in the intestinal loops of rabbit, rat and mice (Ljungh and Kronevi 1982a). Enterotoxin can be produced in synthetic media at the late logarithmic phase of growth (Wadstrom *et al.* 1976b). Its isoelectric point and molecular weight have been estimated to be 4.0-5.7 and 15,000-20,000 respectively (Ljungh *et al.* 1978). It is stable at wide range of pH between 4.5 and 10 but mostly active at alkaline condition (Duby and Sanyal 1978). Enterotoxin is completely destroyed by an enzyme-like papine but it is partially destroy by trypsin or pronase (Ljungh *et al.* 1981). *Aeromons* strains can loose enterotoxigenic potential when stored at laboratory condition but repeated passage in RIL can help to regain enterotoxigenic potential (Annapurna and Sanyal 1977). This observation was also confirmed by Ljungh and Kronevi (1982a). Plasmid was not detected in strains of enterotoxigenic *Aeromonas.* It indicates that production of *Aeromonas* enterotoxin is not plasmid-mediated (Cumberbatch *et al.* 1979).

Enterotoxin of *Aeromonas* spp. inhibits steroid synthesis in Y1 adrenal cell-line (Ljungh *et al.* 1982b). This toxin increases the intracellular contents of cyclic-adenosisn monophosphate (cAMP) without affecting the cyclic-guanosin monophosphate (cGMP) (Ljungh and Wadstrom 1979). Similarly, in RIL, *Aeromonas* enterotoxin increased the contents of cAMP (Duby *et al.*1980). Prostaglandin inhibitors, such as indomethacin and phenylbutazone, usually inhibits cholera toxin-

induced fluid secretion, did not interfere *Aeromonas* toxin induced fluid secretion in the RIL (Duby *et al.* 1980, Ljungh and Kronevi 1982a). On the other hand, chlorpromazine, which usually inhibits both cholera toxin, *E. coli* heat-labile (LT) and heat-stable (ST) toxins, inhibits about 40% less fluid secretion in the *Aeromonas* enterotoxin-treated intestine of mice and rats (Ljungh and Kronevi 1982a).

Aeromonas enterotoxin, like *E. coli* enterotoxin, induces fluid secretion in RIL relatively faster than cholera toxin (Ljungh and Kronevi 1982a). There is no significant difference of electrolyte and albumin contents in fluid secreted in RIL treated with cholera toxin and *Aeromonas* enterotoxin (Streinberg *et al.* 1975, Ljungh and Kronevi 1982a). Six-hour exposure of cholera toxin (Polotsky *et al.* 1977) and enterotoxin of *Aeromonas* in RIL (Annapurna and Sanyal 1977) induced fluid secretion without mucosal damage. These results suggest that *Aeromonas* enterotoxin-induced secretion is mediated by cAMP.

1.2.7.1. Classification of *Aeromonas* enterotoxins:

Aeromonas enterotoxins have been classified into cytotonic and cytotoxic (cytolytic) based on their effect on Y1 and HeLa cell-line. Keusch and Donta (1975) initially proposed this classification.

1.2.7.1.1. Cytotonic enterotoxin (CE): Purification of aeromonad CE was performed by Duby *et al.* (1980) but they failed to calculate its molecular weight and isoelectric point. Subsequently, nature of CE was characterized by Ljungh *et al.* (1981, 1982b). They separated *A. hydrophila* enterotoxin from hemolysin by isoelectric focusing and gel filtration. This toxin showed rounding of cells (without

killing), stimulation of cAMP synthesis and steroid secretion by Y1 cell-line and stimulation of fluid accumulation in RIL. All these characteristic properties of *A. hydrophila* enterotoxin corresponded with the properties of CE initially proposed by Keusch and Donta (1975). The molecular weight of the factor associated with CE of *A. hydrophila* is 15,000 dalton and the isoelectric point was between 4.2 and 4.6. Biological activity of this toxin was not affected when heated at 56°C for 10 minutes. CE of *A. hydrophila* had no antigenic relationship with cholera toxin (Ljungh *et al.* 1981, Ljungh *et al.* 1982b). CE of *Aeromonas* was further characterized by cloning this gene from *A. hydrophila* into *E. c*oli. Enterotoxin from this cloned gene could elongate CHO cell-line and induced fluid accumulation in RIL. These properties were not affected when this toxin was heated at 56°C for 20 minutes. This toxin was also distinct from cholera toxin and *E. coli* enterotoxin (Chakraborty *et al.* 1984).

1.2.7.1.2. Cytotoxic enterotoxin: Other properties of enterotoxins of *Aeromonas* satisfy all the criteria of cytotoxic enterotoxin as defined by Keusch and Donta (1975). These properties are: cell rounding before death and fluid accumulation in RIL. Cytotoxic nature of *Aeromonas* enterotoxin was initially reported by Cumberbatch *et al.* (1979) and Johnson and Lior (1981). Subsequently, Asao *et al.* (1984) purified enterotoxin of *A. hydrophila* and studied its biological activity to reconfirm its cytotoxic nature. It was found that this toxin was hemolytic and induced cytotoxic activity in Vero cell-line and fluid accumulation in RIL. This toxin had molecular weight of 50,000 dalton, isoelectric points of 5.43, 5.41, 5.28 and 5.58. It was inactivated by heating at 56°C for 5 mininutes (Asao *et al.* 1984). This toxin was subsequently proved to be ß-hemolysin (Stelma *et al.* 1986).

1.2.7.1.3. Other toxins: In addition to cytotoxin and cytotonic enterotoxin, aeromonads produce cholera toxin cross-reacting factors and Shiga-like toxins. These toxins are also considered as pathogenic factors.

1.2.7.1.3.1. Cholera-toxin cross-reacting factors: Cholera antitoxin occasionally neutralized the biological activity of enterotoxin of few isolates of *Aeromonas* spp. This enterotoxin is not a real cholera toxin. Therefore, Campbell and Houston (1985) coined the term "Cholera toxin cross-reacting factor (CTC)" to designate this type of enterotoxin of aeromonads. Research teams from Japan (Shimada *et al.* 1984, Honda *et al.* 1985), Australia (James *et al.* 1982) and USA (Campbell and Houston 1985) reported this type of enterotoxin from *Aeromonas*. CTC had molecular weight of 52,000 dalton. Its biological activity is of cytotoxic in nature rather that cytotonic.

1.2.7.1.3.2. Shiga-like toxin: Shiga-like toxin (SLT) is one of the virulence factors associated with enteric infection. Other than *Shigella dysenteriae* (O'Brien *et al.* 1982), SLT has been detected in *E. coli* (Cleary *et al.* 1985) and clinical and environmental isolates of *Vibrio cholerae* and *V. parahaemolyticus* (O'Brian *et al.* 1984). SLT was detected in the environmental and diarrheal isolates of *Aeromonas* spp. (Haque *et al.* 1996). Homologous SLT gene of *E. coli* was identified in the plasmid DNA isolated from three *Aeromonas* strains (Haque *et al.* 1996).

1.2.8. Hemolysins: Initially, Caselitz (1966) suggested two distinct types of hemolysins produced by strains of *Aeromonas*. Subsequently, Wadstrom *et al.* (1976b) and Ljungh *et al.* (1981) confirmed two types of hemolysin produced by this organism. These were α and β-hemolysins.

1.2.8.1. α-hemolysin: It is one type of hemolysin, which induced incomplete lysis of erythrocytes on blood agar plate. Radio-labeled fibroblast cells released large molecular weight markers when exposed to α-hemolysisn. This hemolysin also induced rounding of cells and fading out of nuclei of the fibroblast cells (Thelestam and Ljungh 1981). β-hemolysisn induces morphological changes and membrane permeability of cells are reversible characters. The action of this toxin is restricted to the membrane. However, the role of α-hemolysin is not very clear in the pathogenesis of *Aeromonas* spp. associated diarrhea (Ljungh and Wadstrom 1985).

1.2.8.2. β-hemolysin: It is second type of hemolysin, which induces complete lysis of erythrocytes on blood agar plate. It is heat-labile in nature and toxic to a vatiety of cell-lines, such as CHO, Y1 adrenal and HeLa (Ljungh and Wadstrom 1985). Cytoplasm becomes vaculated and the nuclei were distinctly visible when the cells had been exposed to β-hemolysin (Boulanger *et al.* 1977). β-hemolysin induced release of low molecular weight markers from labeled fibroblast cells and retained marker of high molecular weight (Thelestam and Ljungh 1981).

β-hemolysin can equally bind with glycoproteins of human and murine erythrocytes (Howard and Buckley 1982). Lysis of glycoprotein deficient cell took

places as a result of interaction of β-hemolysin with lipid bilayers. Furthermore, β-hemolysin is lethal to small laboratory animals (Ljungh and Kronevi 1982a). Purified β-hemolysin failed to induce fluid accumulation in RIL but a large amount of β-hemolysin induced hemorrhagic necrocis, edema, neutrofil infiltration etc. Besides, β-hemolysin induced secretion of albumin and calcium contents as a result of mucosal injury (Ljungh and Kronevi 1982a). Purified β-hemolysin inhibited the synthesis of steroid and cAMP in Y1 adrenal and other intestinal cell-lines (Ljungh *et al.* 1982b). Finally, Ljungh and Wadstrom (1985) concluded that neither α nor β-hemolysin is a virulence factor in dirrhral disease.

Pathogenic properties of β-hemolysin were elucidated by Stelma and co-workers (1986). They showed fluid accumulation in RIL by 16 of 19 culture filtrautes (CF) from β-hemolytic *A. hydrophila* strains but CF from non-hemolytic strains of this bacterium failed to induce fluid accumulation in RIL. Thus, Stelma *et al.* (1986) concluded that β-hemolytic strains of *A. hydrophila* are capable of inducing gut permeability in absence of cytotoxic enterotoxin.

More evidencce in support of β-hemolysin as a pathogenic factor came from a recent work of Wang *et al.* (1999). This group has discovered receptor for *A. sobria* β-hemolysin on the surface of human intestinal embryonic cell-line known as 407 cell-line and subsequently claimed that β-hemolysin plays an important role in the pathogenesis of human diarrhea.

1.2.8.3. CAMP-hemolysin: The term CAMP or CAMP-like test was used for designating synergistic hemolytic activity of *Staphylococcus aureus*. This term has

been coined from the initial letters of the names of three pioneer authors: Christie, Atkins and Munch-Petersen (1944). CAMP-hemolysin is an enzyme known as sphingomyelinase C that hydrolyze sphingomyelin. It is also known as β-toxin of *S. aureus*. Sphingomyelinase C of *S. aureus* can interact with chelators (Smyth *et al.* 1975), phospholipase C of *Bacillus cereus* (Colley *et al.* 1973) and *Listeria monocytogenes* (Skalka *et al.* 1982) to cause complete lysis of erythrocyte.

Detection of CAMP-hemolysin was used for differentiating motile mesophilic *Aeromonas* spp. (Figura and Guglielmetti 1989). Presence or absence of oxygen during incubation affected the production of CAMP-hemolysin in different species of *Aeromonas* (Altwegg *et al.* 1990). Detection of CAMP-hemolysin was thought to be useful markers to differentiate *A. hydrophila* and *A. veronii* bv sobria (both positive) from *A. caviae* (negative) (Carnahan *et al.* 1991c). Now it is evident that detection of CAMP-hemolysin cannot be used for differentiating any one of the three species (Gubash 1997). The nature of CAMP-hemolysin of *Aeromonas* is still unknown. However, the role of CAMP-hemolysin in the pathogenicity of *Aeromonas* has not been elucidated.

1.2.9. Bacteriocin: Extracellular bacterial metabolites occasionally inhibit the growth of same or related bacteria. These antagonistic bacterial metabolites are known as bacteriocin (Puma *et al.* 1990, Lindquist and Emilson 1991, Mechan *et al.* 1991). Bacteriocins are proteins elaborated by different bacterial species. It differs with respect to chemical properties, host range and mode of action (Hardly 1975, Tagg *et al.* 1976. Konisky 1982). Excretion of bacteriocin may be a natural phenomenon for

suppressing one bacterial population by others for a particular ecological niche(s). Bacteriocin production of *Aeromonas* has been reported (Sundraraj *et al.* 1994). *Aeromonas* bacteriocin shows antagonistic effect against *Vibrio cholerae* non- O1, *Escherichia coli* and homologous strains of *Aeromonas* spp.

Enzymes: Other than enterotoxins, aeromonads produce different enzymes associated with virulence (Holder and Haidaris 1979, Janda and Bottone 1981). Waltman *et al.* (1982) investigated the ability of strains of *A. hydrophila* isolated from different sources to produce varieties of enzyme using the API 20 zym system. They were able to detect different enzymes, such as caprylate esterase-lipase, leucine amino peptidase, acid phosphatase, phosphoamidase, and N-acetyl-β-glucosaminidase. Isolates showed variability with respect to production of other enzymes, such as alkaline phosphatase, butyrate esterase, myristate lipase, trypsin, β-galactosidase, α-glucosidase and β-glucosidase (Waltman *et al.* 1982).

Using API 20 zym kit, Rahim *et al.* (1991) detected 14 different enzymes, such as alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, cystin arylamidase, trypsin, phosphatase acid, phosphoamidase, β-galactosidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosidase, But Rahim *et al.* (1991) could not detect other enzymes, such as chymotrypsin, β-galactosidase, α-glucosidase, α-mannosidase, and α-fucosidase. This observation was consistent with the findings of Ljungh *et al.* (1977) and Waltman *et al.* (1982). However, Rahim *et al.* (1991) showed significant difference among *A. hydrophila* strains isolated from

surface water and fish with respect to three enzymes, such as cystine arylamidase ($P<0.05$), α-glucosidase ($P< 0.01$) and β-glucosidase ($P<0.05$).

1.2.10.1. Protease: Strains of *Aeromonas* spp. consistently produce protease. Production of at least one heat-labile protease has been reported (Riddle *et al.* 1981, Buckley *et al.* 1981) but the role of *Aeromonas* protease has not been elucidated. Experimental fish infection with extracellular metabolites of *Aeromonas* spp. induced lesions similar to natural infection. Extracellular metabolites from protease-deficient mutants of Aeromonas spp. showed more toxicity in fish (Allan and Steven 1981). This indicates that (1) either protease is not a virulence factor or (2) protease of Aeromonas spp. inactivates virulence factor(s) of this bacterium, which are excreted in culture media. Moreover, Hsu and co-workers (1981) correlated the production of elastase and staphylolytic enzymes with the virulence properties of *Aeromonas* spp.

Khashe *et al.* (1996) evaluated the pathogenic potentiality of protease and elastase in low and high virulent strains of *Aeromonas* spp. It was found that highly virulent strains produced eight-fold higher proteolytic activity in liquid medium than moderate to low virulent strain of *Aeromonas*. Similarly, elastase activity was also prominent in highly virulent strains than low to moderately low pathogenic strains of *Aeromonas*. Chang *et al.* (1997) cloned the gene-encoding extracellular protease from *A. hydrophila* to study the gene at a molecular level.

1.2.10.2. Other enzymes: Several other enzymes, such as pyraminidase (Carnahan *et al.* 1990, Altwegg *et al.* 1991), serine protease (Huselein *et al.* 1991, Rivero *et al.* 1991), lipase (Chuang *et al.* 1997), metaloprotease (Toma *et al.* 1999, Kawakami *et*

al. 2000), arylamine N-acetyltransferase (Chung *et al.* 1998), phosphoamidase (Merino *et al.* 1999), elastase (Cascon *et al.* 2000) etc.

1.2.11. Siderophores: Bacterial growth requires soluble iron as one of the essential nutrients required for many biological processes of the organism including electron transport chain and also as co-factor of enzymes. (Neilands 1981). At iron-limiting condition, either in the host or in the environment, bacteria produce iron-chelating compounds of low molecular weight. These compounds are known as siderophores (Neilands 1981). Thus, siderophores act as extracellular agents for solubiliging iron from minerals and organic compounds under iron-limiting condition. Bacterial cell possesses specific membrane receptors and transport system which recognize iron-siderophore complex for internalization and subsequent use by microorganisms (Ames 1986). Crosa (1989) has reviewed a detailed molecular mechanism of siderophore production.

Different bacteria produce siderophores (Neilands 1981). Similarly, amnobctin, a new siderophore has been reported from *A. hydrophila*. (Barghouhti *et al.* 1989). This new siderophore is produced in two forms: amnobactin T that contains tryptophan and amnobactin P that contains phenylalanin. Biologically active both amnobactin T and P are produced simultaneously throughout the life-cycle of *A. hydrophila*.

Clinical (diarrheal) and environmental (pond water) isolates of *A. hydrophila* can produce siderophores. Clinical isolates produce higher quantities of siderophores than environmental isolates. Production of siderophores is not affected after curing the plasmid from the host strains (Naidu and Yadav 1997). Different fish isolates of

Aeromonas produce different quantities of siderophore and hemolysin (Santos *et al.* 1999). At iron-limiting condition, some strains of *Aeromonas* produce proteins of different molecular weight in addition to siderophores. These proteins are not produced normally in iron-containing media (Alavandi and Ananthan 2000).

1.2.12. S-layer: Paracrystaline layer, which lies to the periphery of outer membrane of the bacterial cell wall, is known as surface layer or S-layer. It is composed of different protein sub-units and can be readily identified in thin sections of bacterial cells examined under transmission electron microscopy. Strains having S-layer, autoagglutinate or aggregate in broth after boiling (Kokka *et al.* 1991). S-layer has been detected in A. *hydrophila* and A. *hydrophila* bv veronii (Paula *et al.* 1988, Kokka *et al.* 1991). Other pathogenic microorganisms having S-layer are: *Clostridium botulinum*, *A. salmonicida*, and *Campylobacter fetus* subspp. Fetus, *Mycobacterium bovis*, and *Campylobacter* (Wolinella) *recta* (Messner and Sleytr 1992, Kobayashi *et al.* 1993) but the role of S-layer in the pathogenesis of *Aeromonas* has not been elucidated.

S-layer provides protection against *Aeromona* strains from bactericidal activity of 65% pooled human serum (PHS). These strains are moderately pathogenic to mice. Strains with S-layer possess defined lypopolysaccharide (LPS) patterns. On the other hand, S-layer negative strains are susceptible to complement-mediated lysis and is low pathogenic to mice. S-layer negative strains possess distinct O-polysaccharide side chain, which is clearly visible in silver stained LPS (Paula *et al.* 1988).

Review of above-mentioned literature on *Aeromonas* spp. suggests that this organism is distributed in diverse ecological niches. They are important pathogens of different animals, including human beings. In Bangladesh, very limited *Aeromonas*-related studies have been conducted. *Aeromonas*-related studies of Bangladesh have mostly focused on the isolation of *Aeromonas* spp. from water samples of pond (Islam *et al.* 1992), river and lake (Parveen *et al.* 1995), diarrheal patients and healthy controls (Hossain *et al.* 1991), infected fish (Rahin *et al.* 1984, 1985) and prawns (Rahim and Aziz 1994). However, there was no attempt to study the impact of physicochemical parameters of water on density of *Aeromonas*, detection of *Aeromonas* phages and pili and evaluate the pathogenic potential of cytotoxic enterotoxin gene-positive and gene-negative stsrains of *Aeromonas* spp. isolated form the water and duckweed (aquatic plant) and fish samples.

1.3. OBJECTIVES

It is evident from the forgoing review of literature that *Aeromonas* is an important pathogen, which is associated with intestinal and extra-intestinal infections of human beings. It can infect domestic and aquatic animals. The density of *Aeromonas* spp. fluctuates with variation of physicochemical parameters of water. Human beings and aquatic animals, including fish, acquire *Aeromonas* infection from polluted waters. *Aeromonas*-infected fish shows disease symptoms, such as furunculosis, red-sore disease, ulcerative disease syndrome etc. Hatchery water infested with *Aeromons* induces mortality of fingerlings and results in huge economic losses in the fish industry. Thus, it is important to monitor and control growth of *Aeromonas* spp. in water of fish farms. Therefore, the present study has been undertaken with the following objectives:

a) to assess the abundance of *Aeromonas* spp. in aquatic plant, water and gills and intestinal contents of fish of both wastewater and non-wastewater areas.

b) to monitor physicochemical parameters of water and correlate it with the density of *Aeromonas* spp. in aquatic plant, water and gills and intestinal contents of fish of both wastewater and non-wastewater areas.

c) to speciate isolates of aeromonads from aquatic plant, water and gills and intestinal contents of fish.

d) to test hemolytic activity of isolates of *Aeromonas* spp.

e) to measure the density of *Aeromonas* phages in water and sediment samples of sewage contaminated and uncontaminated ponds.

f) to detect cytotoxic enterotoxin (*act*) gene of isolates of *Aeromonas* spp.

g) to detect enterotoxicity, cytotoxicity, hemolytic activity, CAMP-like hemolysin and drug resistance among *act* gene-positive and gene-negative isolate of *Aeromonas* spp.

h) to study the factors affecting production of cytotoxic enterotoxin (Act) by a strains of *A. sobria* harboring *act* gene.

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CHAPTER - 2

DISTRIBUTION OF *AEROMONAS* SPP. IN AQUATIC PLANT, WATER AND FISH

2. DISTRIBUTION OF *AEROMONAS* SPP. IN AQUATIC PLANT, WATER AND FISH

2.1. INTRODUCTION

Aeromonas species are autochthonous flora of aquatic environments. Species belonging to this genus are distributed in diverse ecological niches. In aquatic ecosystems, density of *Aeromonas* varies with seasonal variation (Fliermans *et al.* 1977). Strains of *Aeromonas* spp. have been isolated from brackish water (Kaper *et al.* 1981), fresh water (Rippey and Cabelli 1979), domestic water (Burke *et al.* 1984a, 1984b), wastewater (Hassani *et al.* 1992), stabilization pond (Boussaid *et al.* 1991), wastewater purification plants (Stecchinis and Domenis 1994), intestinal contents of riverine fish (Sugita *et al.* 1995) etc. *Aeromonas*-associated infections of various aquatic animals such as bottle-nosed dolphin (Cusic and Bullock 1973), cod fish (Larsen and Jansen 1977), alligators (Shotts *et al.* 1972, Gorden *et al.* 1979) and its isolation from healthy and moribund fish (Leblance *et al.* 1981) have been reported. Moreover, *Aeromonas* spp. can cause ulcerated fish disease (Rahim *et al.* 1984) and septicemia in gold fish (Merino *et al.* 1988). *Aeromonas* was suspected as the probable agent of causing fish epizootic of Thailand in 1982 (Sukroongreung *et al.* 1983). Thus it is one of the important fish pathogens and can adversely affect pisciculture.

Physicochemical parameters of water affect distribution of aeromonads in an aquatic environment (Kaper *et al.* 1981). Occurrence of aeromonads in chlorinated drinking water has been reported from Canada (Clark *et al.* 1982), the United States of America (Le Chevallier *et al.* 1982) and India (Sen and Jacob 1969). Isolation of *Aeromonas* spp. was also reported from chlorinated and unchlorinated water supply of Australia (Burk *et al.* 1984a, 1984b). Presence of *A. hydrophila* in metropolitan water

supply correlated with isolation of this pathogen from diarrheal stools in Australia (Burke *et al.* 1984a).

Aeromonas is one of the important agents associated with diarrhea (Kuijper *et al.* 1991, Teka *et al.* 1999). *Aeromonas* spp. associated diarrhea is usually mild and self-limiting (Holmberg and Farmer 1984). However, cholera-like watery diarrhea (Champsaur *et al.* 1982) as well as dysentery-like syndrome (Rahman and Willoughby 1980) are associated with aeromonad infection. Besides, *Aeromonas* spp. have been incriminated to be associated with wound infection (Kienzle and Muller 2000), arthritis (Roux *et al.* 2000) and corneal ulcer (Carta *et al.* 1994). This bacterium can cause life-threatening human diseases, such as septicemia (Tabata *et al.* 1999), meningitis (Lin and Cheng 1998), hemolytic-uremic syndrome (Fang *et al.* 1999) etc.

Aeromonas spp. produce various virulence-associated extracellular metabolites, such as enterotoxin (Chopra and Houston 1999), cytotoxin (Alavandi *et al.* 1999), hemolysin (Fujii *et al.* 1999) etc. Cytotoxin gene of *A. hydrophila* has been cloned and conclusively proved its role in the pathogenicity of enteric infection (Chakraborty *et al.* 1984). Besides, *Aeromonas* spp. produce other putative virulence-associated factors, such as straight (Sato *et al.* 1989) and flexible pili (Ho *et al.* 1990), collagen-binding protein (Gullberg *et al.* 1989), hemagglutinin (Majeed and Macrae 1994). The pathogenic role of all these factors has been proved experimentally.

In Bangladesh, strains of *Aeromonas* spp. have been isolated from ulcerated fish (Rahim *et al.* 1984), various components of a pond ecosystem (Islam *et al.* 1992), freshwater prawn (Rahim and Aziz 1994), lake and river (Parveen *et al.* 1995) etc. The present study was conducted in a duckweed-based pisciculture farm having two

areas: wastewater and non-wastewater. DW grown in the wastewater stabilization ponds was used as feed in the fish ponds of wastewater area. In the non-wastewater area, DW grown by chemical fertilizer was used as feed in the fish ponds of non-wastewater area. To evaluate *Aeromonas*-associated hazards of wastewater grown DW as fish feed in pisciculture, the abundance of *Aeromonas* in DW, water and gills and intestinal contents of fish of both the areas were compared. Furthermore, the incidence of fish-disease and correlation of physicochemical parameters of water with the abundance of *Aeromonas* in water, DW, gills and intestinal contents of fish were also studied.

2.2. MATERIALS AND METHODS

2.2.1. Sampling sites and schedule: The sampling sites were located within the Kumudini Hospital campus of Mirzapur Thana, Tangail district of Bangladesh. It is approximately 60 km to the north of Dhaka, the capital city of Bangladesh. The pisciculture farm had two areas: wastewater and non-wastewater. In the first week of every month, DW (*Lemna minor*), water and fish samples were collected for a period of 12 months from August 1994 through July 1995 from both the areas for microbiological analysis of *Aeromonas* spp.

2.2.2. Wastewater area: One primary stabilization pond (60m x 60 m, 0.9 m depth), six DW-based wastewater treatment ponds (575m x 9m, 0.9 m depth each) and three fish ponds (60m x 60m, 0.9m depth each) were located in this area. Six DW-based wastewater treatment ponds were dug and connected in a zigzag fashion to ensure slow flow of wastewater into these ponds. Untreated wastewater was pumped into the first DW-based wastewater treatment pond to ensure gradual flow up to the sixth pond, which was connected to a river through a 3.0 inch plastic pipe (Fig. 2.1). When

considered safe from chemical and bacteriological point of view, treated wastewater was allowed to pass into the river. From the wastewater area, samples were regularly collected from the untreated wastewater pond, three of six DW-based wastewater treatment ponds and three fish ponds for microbiological analysis of aeromonads.

2.2.3. Non-wastewater area: Four fish-growing ponds and 59 DW growing ponds (9.5m x 60m each) were located in this area (Fig.2.1). Chemical fertilizer-nourished DW was grown in these ponds. DW was deposited into the fish ponds as fish-feed. Two of the 59 DW-growing ponds and two of the four fish-growing ponds were arbitrarily selected from the non-wastewater area for Isolation of *Aeromonas* spp.

2.2.4. Sample collection: 500 ml of water was collected in presterilized Nalgene plastic bottles from two of the 59 DW-growing ponds and from two of the four fish-growing ponds of non-wastewater area. An equal volume of water was collected in a similar container from the untreated wastewater pond, three of six DW-based wastewater treatment ponds and two fish ponds of the wastewater area. Five hundred g (wet weight) of DW was collected in polyethylene bags from two of the 59 DW-growing ponds and from two of the four fish ponds of the non-wastewater area. The same quantity of DW was collected in similar containers from three of the six wastewater treatment ponds and three fish ponds of the wastewater area. From the fish ponds of both wastewater and non-wastewater areas, DW was collected within one hour of depositing them in the ponds as fish feed.

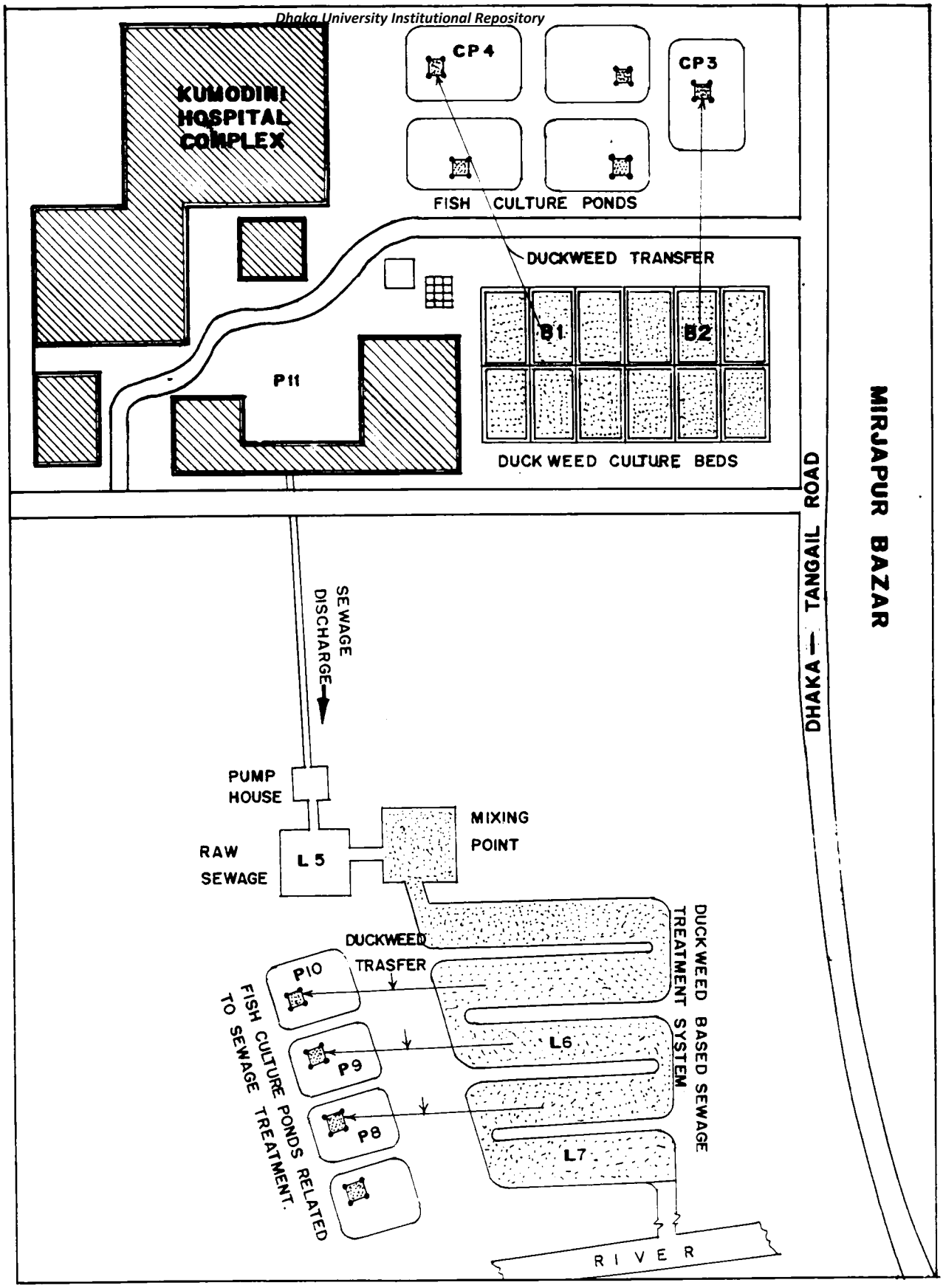


Fig. 2.1. Schematic diagram of sampling sites at Mirzapur, Tangail

Fish was sampled manually by fishing net. Approximately, 750 g (wet weight) of either tilapia (*Oreochromis nilotica*) or silver carp (*Ctnopharyngodon idella*) was sampled from the selected ponds and taken in polyethylene bags.

2.2.5. Measurement of physicochemical parameters: Of various physicochemical parameters, a mercury thermometer was used for measuring temperature on the spot. pH and electrical conductivity were measured in the laboratory using digital pH meter (Orion Research, digital pH-millivolt meter 611) and conductivity meter (DREL/5 HACH) respectively.

2.2.6. Transportation of samples: Immediately after collection, DW, water and fish samples were taken in an insulated box with cool packs. The box containing samples was transported to the laboratory and the processing of samples was completed within 4 to 6 hours after collection following the standard procedure (APHA 1991).

2.2.7. Processing of samples: DW samples were washed in presterilized normal saline (0.85%) before processing to eliminate loosely attached bacteria. Ten g of washed DW sample was homogenized in 90 ml of normal saline in an electrical blender (Waring Commercial blender, New Hartford, Connecticut, USA) for one minute.

Gill-covers of the fish samples were split open and the exposed gills were washed in normal saline to eliminate loosely attached bacteria. The ventral part of the fish was then cut open with sterile scissors and washed with sterile normal saline before dissecting parts of the intestine. Each of the ten g washed gills and intestinal contents was separately homogenized in 90 ml normal saline in an electrical blender

for one minute. The cup of the blender was sterilized in boiling water for 10 minutes after processing each sample.

Water, homogenized DW, gills and intestinal contents of fish were serially diluted separately in sterile normal saline from 10^{-1} to 10^{-5}. 25 µl sample from each dilution was plated on MacConkey agar plate in duplicate following the drop plate technique (Hoben and Somasegaran 1982). 50 µl of undiluted water sample or similar amount from each dilution (serially diluted as above when required) was plated on MacConkey agar plate (Rahim *et al.* 1994) in duplicate following drop plate technique (Hoben and Somasegaran 1982). Inoculated plates were incubated at 37°C for 18-24 hours. Following incubation, characteristic viable aeromonad colonies (3-4 mm in diameter, oxidase positive, non-lactose fermenting colonies with smooth surface, Rahim and Aziz 1994) were counted from each plate as suspected aeromonads and the counts were converted into aeromonad density as colony forming unit (cfu) per ml water or per g homogenized DW, gills and intestinal contents of fish.

2.2.8. Speciation of aeromonads: Three to five typical aeromonad colonies were speciated using conventional biochemical reaction (Popoff 1984). These included sensitivity to O/129 (2,4-diamino, 6,7-diisopropyl pteridine, Sigma), dehydrogenation of arginine, decarboxylation of lysine and ornithine, utilization of arabinose and salicin, hydrolysis of esculin, Voges-Proskauer reaction and growth in peptone-water containing 0.3 and 6.0% NaCl (Popoff 1984).

2.2.9. Test of hemolytic activity: The hemolytic activity of aeromonads was tested on blood agar plate containing 5% defibrinated sheep-blood (Rahim and Aziz 1994). Test isolates were streaked onto the blood agar plate and incubated aerobically at 37°C for 18-24 hours. Following incubation, hemolytic strains showed prominent

zone of hemolysis around the colony and were categorized as α, β and γ (Rahim and Aziz 1994) depending on the type and extent of hemolytic activity of the isolate.

2.2.10. Stool culture of duckweed and fish handlers: Stool samples of four handlers of fish and DW each from wastewater and non-wastewater areas were collected on the same day of sampling. The handlers were also interviewed for any history of diarrhea. Stool samples were cultured for vibrios, salmonellae, shigellae, aeromonads (Aziz *et al.* 1986) and *Campylobacter* (Blaser *et al.* 1980) following the standard procedure as described by Stoll *et al.* (1982).

2.2.11. Data Analysis: The density of *Aeromonas* species was log-transformed to avoid the asymmetry of density distribution. The data were then analyzed using SPSSwin (SPSS Inc. Chicago). Correlation-coefficient was obtained to find any significant interrelationship among the variables.

2.3. RESULTS

2.3.1. Percentage reduction of *Aeromonas*: Geometric mean of density of *Aeromonas* in the raw sewage and in the duckweed treated wastewater was $5.3x10^4$ and $9.9x10^1$ colony-forming units per ml (cfu/ml) respectively during the entire period of investigation (Table-2.1). The decrease in *Aeromonas* counts was 5.3×10^4 cfu/ml, which corresponds to 99.8% reduction in *Aeromonas* population. Significant difference ($P<0.001$) was found between *Aeromonas* density in water of raw sewage and that in treated stabilization pond. There was no significant difference between *Aeromonas* density in winter and summer. *Aeromonas* density reduction was higher in Winter compared to Summer.

2.3.2. Concentration of aeromonads in wastewater and non-wastewater areas: During the study period, different kinds of sample, such as DW, water and fish samples were collected monthly from the wastewater and non-wastewater areas. Samples were processed for the determination of density of aeromonads on MacConkey agar plate. In the wastewater areas, the counts of aeromonads in DW, water, gills and intestinal contents of fish ranged between $6.0x10^3$ and $1.6x10^9$, between $2.0x10^1$ and $1.5x10^4$, between $1.0x\ 0^4$ and $3.0x10^7$ and between $1.2x10^5$ to $2.2x\ 0^9$ respectively. In the non-wastewater area, the density of aeromonads in DW, water, gills and intestinal contents of fish ranged between $2.0x10^1$ and $8.0x10^8$, between $1.0x10^1$ and $8.0x10^2$, between $2.0x10^3$ and $4.0x\ 0^8$ and between $1.2x10^6$ and $2.8x10^{10}$ respectively. In wastewater area, the highest aeromonad density was detected in untreated wastewater pond throughout the study period. In the fish sample, aeromonad density was higher in the intestinal contents than gills in both the areas.

The sampling sites of both wastewater and non-wastewater areas were very close. Therefore, *Aeromonas* density in each component of the respective area has been expressed as average for comparison. The average density (cfu per g or ml) of aeromonads isolated from DW, water and fish (gills and intestinal contents) of these two areas are shown in Figs. 2.2, 2.3 and 2.4 respectively. There was no significant difference between wastewater and non-wastewater areas in terms of the counts of *Aeromonas* in DW (P=0.053), water (P=0.384), gills (P=0.402) and intestinal contents (P=0.312) of fish.

2.3.3. Speciation of aeromonads: One hundred and eighty-nine isolates were identified to the species level. Of the various species, 28% of *A. hydrophila* (53 of 189), 56.6% of *A. sobria* (107 of 189) and 15.3% of *A. caviae* (29 of 189) were

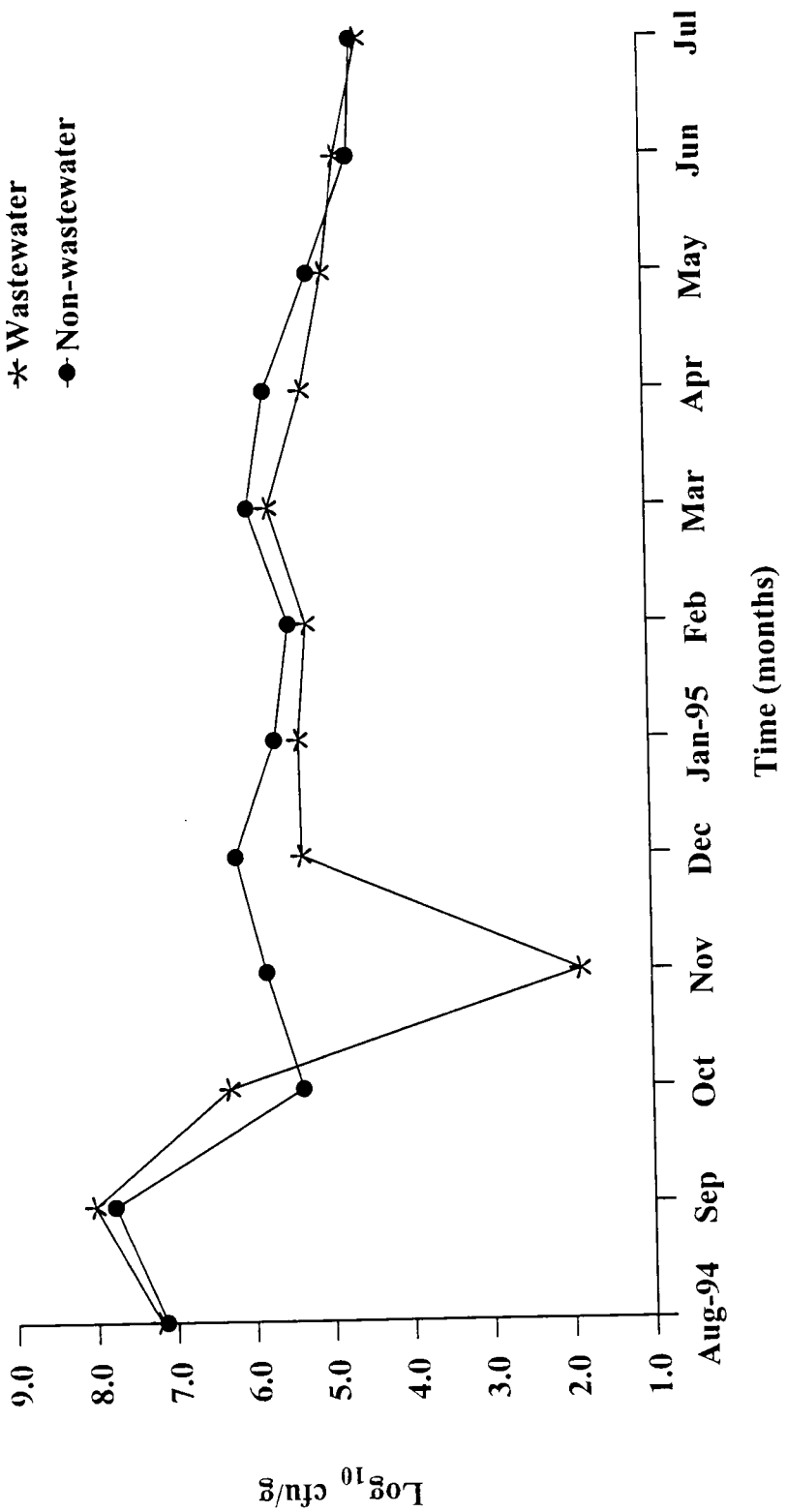
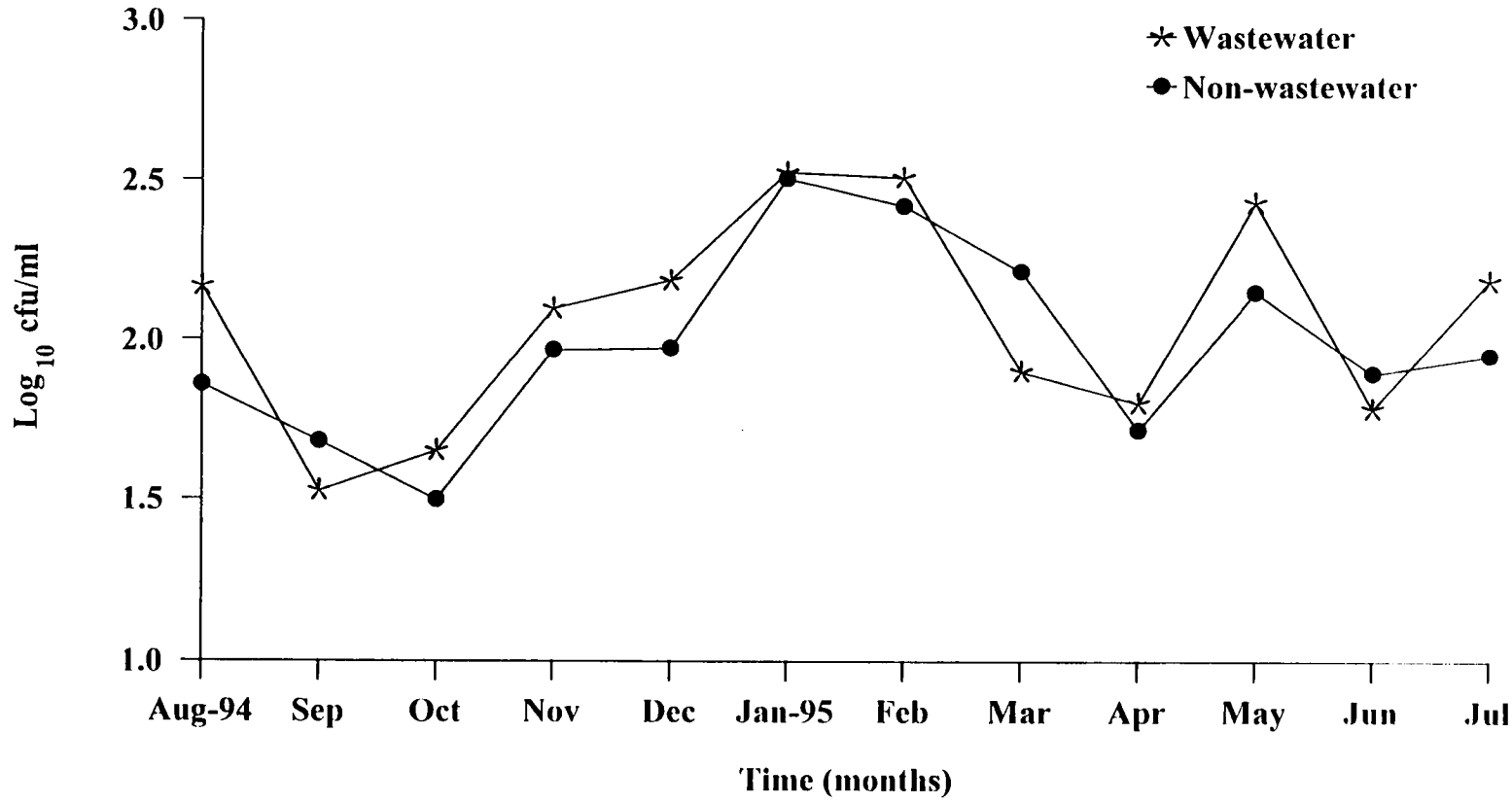


Fig. 2.2. *Aeromonas* density in duckweed samples of wastewater and non-wastewater areas

Fig. 2.3. *Aeromonas* density in water samples of wastewater and non-wastewater areas



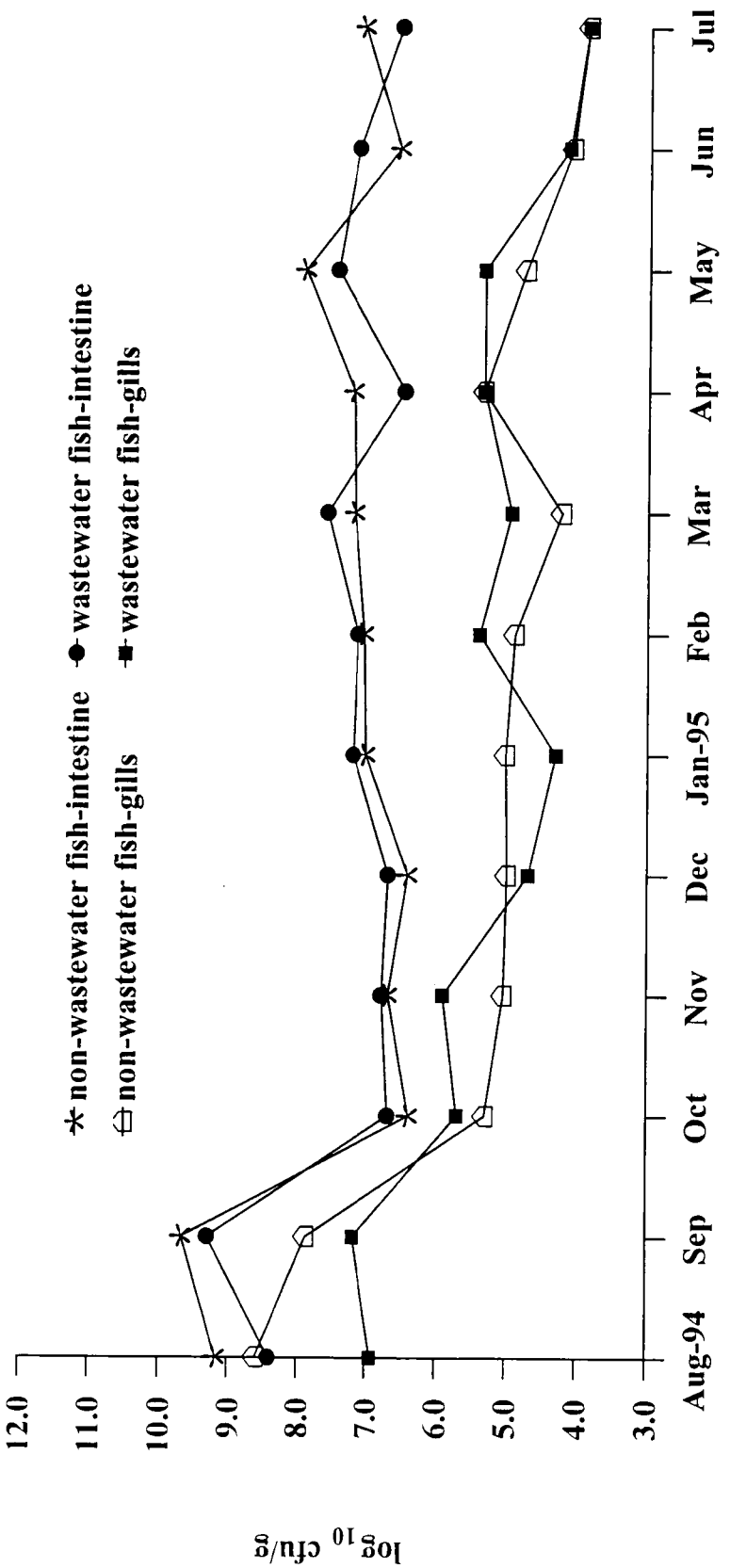


Fig. 2.4. *Aeromonas* density in gills and instestinal contenents of fish in wastewater and non-wastewater areas

recovered. Of the various samples, the highest incidence of *A. hydrophila* and *A. sobria* was recorded in the water samples. DW samples demonstrated the highest incidence of *A. caviae*. The abundance of different species in different sample is shown in Table-2.2.

2.3.4. Hemolytic activity: One hundred and eighty nine isolates of aeromonads were tested for hemolytic activity. Of these isolates (n=189), hemolytic activity ($\alpha+\beta$) was more among *A. sobria* isolates of water (13.2%) and fish-gills (12.7%) than fish-intestinal contents (10.5%) and DW (9.5%). The detailed hemolytic activity of different aeromonads and their source of isolation are shown in Table-2.2.

2.3.5. Physicochemical parameters:

Temperature (°C) of water in both wastewater and non-wastewater areas ranged between 18° (Winter) and 35°C (Summer). *Aeromonas* density of gills and intestinal contents of fish significantly correlated with temperature of water in both wastewater ($P<0.001$) (Table-2.3) and non-wastewater ($P<0.01$) areas (Table-2.4).

pH of water in wastewater and non-wastewater areas ranged between 6.9 and 8.4 and between 6.7 and 8.7 respectively. In non-wastewater area, pH of water significantly correlated with *Aeromonas* density in gills ($P<0.001$) and intestinal contents ($P<0.001$) of fish (Table-2.4).

The electrical conductivity (millisiemens/meter = mS/m) of water samples in wastewater and non-wastewater areas ranged between 31.0 (wastewater pond no. 2, July 1995) to 115.0 mS/m (primary stabilization pond, February 1995) and between 40.0 (DW growing pond no. 1 and 2, May 1995) and 54.5 mS/m (fish pond no. 2, February 1995) respectively. Conductivity of water in wastewater area significantly correlated with the *Aeromonas* density in water ($P<0.001$) (Table-2.3). Similarly,

Aeromonas density of water significantly correlated with the density of *Aeromonas* in gills ($P<0.001$) and intestinal contents ($P<0.001$) of fish of non-wastewater area.

2.3.6. Stool culture: During the study period, none of the DW handlers of both wastewater and non-wastewater areas had any complaint of diarrhea. Besides, none of the enteric pathogens was isolated from stool samples of persons handling fish and DW of either area.

2.3.6. Fish epizootic: None of the fish species showed any sign of superficial infection or lesions on any part of the body. All the fish species remained healthy and fish production was satisfactory.

Table-2.1. Reduction of aeromonad density due to treatment of wastewater by duckweed

Source	Cold[a] period	Warm[b] period	Entire[c] period
Raw sewage	$6.3x10^{3d}$	$2.3x10^{3}$	$5.3x10^{4}$
Stabilization pond	$1.1x10^{2}$	$9.2x10^{1}$	$9.95x10^{1}$
Decreased aeromonad density	$6.2x10^{3}$	$4.9x10^{3}$	$5.3x10^{4}$
(% decrease)	(98.3)	(96.0)	(99.0)

[a] Three months from December 1994 through February 1995; [b] Three months from August through October 1994; [c] Twelve month from August 1994 through July 1995; [d] Geometric mean of *Aeromonas* density in water

Table-2.2. Hemolytic activity of *Aeromonas* spp. isolated from different sources

Species	Hemolytic activity of *Aeromonas* spp. (n=189) associated with			
	DW No. (%)	W No. (%)	FG No. (%)	FI No. (%)
A. hydrophila	11 (20.8)	15 (9.4)	7 (13.2)	6 (11.3)
A. sobria	18 (16.8)	25 (23.4)	24 (22.4)	20 (18.7)
A. caviae	3 (10.3)	2 (6.9)	3 (10.3)	4 (13.8)

DW = Duckweed, W = Water, FG = Fish-gills, FI = Fish-intestinal contents.

Table-2.3. Correlation coefficient of *Aeromonas* density in duckweed, water, fish-gills and fish-intestinal contents with physicochemical parameters of water in wastewater area

Aeromonas from	Physicochemical parameters of		
	PH	Temperature	Conductivity
Duckweed	0.1667	0.4393	-0.5019
Water	-0.0803	0.1340	0.8633**
Fish-gills	0.4940	0.7030*	-0.2862
Fish- intestinal contents	0.4451	0.7006*	-0.2852

*P≤0.01, **P≤0.001

Table-2.4. Correlation coefficient of *Aeromonas* density in duckweed, water, fish-gills and fish-intestinal contents with physicochemical parameters of water in non-wastewater area

Aeromonas from	Physicochemical parameters of		
	pH	Temperature	Conductivity
Duckweed	0.6360	0.6372	0.6044
Water	0.3820	0.0270	0.0037**
Fish-gills	0.9872**	0.9874**	0.9644**
Fish-intestinal contents	0.9899**	0.9922**	0.9764**

**P≤0.001

2.4. DISCUSSION

Aeromonas is nutritionally versatile as evident by its ability to grow in water having low concentration of any substances, such as glucose, acetone, glutamate, succinate and lactate (Van der Kooij *et al.* 1980). Besides, various components of biomass, such as long-chain fatty-acids and amino-acids at low concentration can enhance its multiplication in water (Van der Kooij *et al.* 1988). Sewage, being rich in various nitrogenous waste materials, can enhance the growth of *Aeromonas* spp. Possibly for this reason, the density of *Aeromonas* was highest in sewage water throughout the study period. This result is consistent with the findings of Monfort and Baleux (1986), Boussaid *et al.* (1991) and Hassani *et al.* (1992).

The conventional biochemical scheme of Popoff (1984) was followed in this study and three species of *Aeromonas* (*A. hydrophila*, *A. sobria* and *A. caviae*) were identified from various kinds of sample. This is consistent with the past reports from Bangladesh (Islam *et al.* 1992, Rahim and Aziz 1994). However, different species were isolated from different kinds of sample (Table-2.2). But it is difficult to explain the association of a particular species with a particular biotic component of an ecosystem. Further study is needed to confirm the association of a particular species with a particular biotic component of an ecosystem.

In Bangladesh, different species of *Aeromonas* are ubiquitous in river, lake and pond ecosystems (Islam *et al.* 1992, Parveen *et al.* 1995). In the present study, the abundance of *A. sobria* was higher than *A. hydrophila* and *A. caviae* in various components, such as DW, water, gills and intestinal contents of fish. These findings are in agreement with the previous reports from Bangladesh (Islam *et al.* 1992, Parveen *et al.* 1992, Rahim and Aziz 1994). In sewage water of France, Monfort and Baleux (1990) reported the prevalence of *A. caviae* in inflow and *A. sobria* in outflow.

However, in the stabilization pond of an arid region of Morocco, *A. caviae* and *A. hydrophila* were simultaneously predominant in the inflow and only *A. sobria* was predominant in the final effluent (Boussaid *et al.* 1991). This discrepancy in results from different geographical regions is possibly due to the differences in climate and physicochemical parameters of water in different regions of the world.

Hemolysin is one of the virulence factors of *Aeromonas* spp. (Stelma *et al.* 1986). Two types of hemolysins of *Aeromonas* spp. (α and β) were detected in this study. This is consistent with the past reports from Bangladesh (Parveen *et al.* 1992, Rahim and Aziz 1994). Moreover, in this study, β-hemolytic activity was found more in *A. sobria* than in *A. hydrophila* and *A. caviae*. These results are in agreement with those of Imzlin *et al.* (1996). However, Parveen *et al.* (1992) failed to isolate β-hemolytic *A. caviae* from Bangladesh but in the present study 17% (5 of 29) strains of *A. caviae* were β-hemolytic. The prevalence of β-hemolytic *A. caviae* was also reported from France (Monfort and Baleux 1991) and Morocco (Imziln *et al.* 1996) in sewage samples.

In aquatic environment, *Aeromonas* is primarily associated with phyto-and zooplankton (Simidu *et al.* 1971). Aquatic animals including fish that feeds on phyto- and zooplankton may accumulate *Aeromonas* in their gut from the surrounding aquatic ecosystem. In the present study, possibly for this reason, *Aeromonas* concentration in fish (gills and intestinal contents) was higher than in water and DW. Consistent to the present study, it was found that concentration of *Aeromonas* was also higher in the intestine than in other body parts of prawn (Rahim and Aziz 1994).

Aeromonas can grow at a wide range of pH (5.0 to 9.0) but unable to grow at a pH lower than 4.0 and higher than 10.0 (Hazen *et al.* 1978). In this study, pH of water ranged between 6.7 and 8.7. Significant correlation was found between pH of water and *Aeromonas* density in gills and intestinal contents of fish of non-wastewater area (Table-2.4).

Temperature is one of the important factors that affects the growth and distribution of *Aeromonas* spp. in the aquatic environment (Kaper *et al.* 1981). Rouf and Rigney (1971) found that the optimum growth temperature of *Aeromonas* was close to 45°C but most strains of *Aeromonas* can grow at 35°C. In a laboratory-based experiment, Cavari *et al.* (1981) demonstrated elevated metabolic activity at 25°C compared to 4°C and ultimately explained the possible correlation of *Aeromonas* counts with elevated temperature of water in the Anacostia river of USA. In the present study, water temperature ranged between 18 and 35°C and *Aeromonas* counts positively correlated with water temperature (Tables-2.3 and 2.4). This result is in agreement with the previous reports (Hazen *et al.* 1978, Hazen and Fliermans 1979, Rippey and Caballi 1979, Seidler *et al.* 1980, Kaper *et al.* 1981). However, this result was in contrast to the findings of Burke *et al.* (1984a) and Boussaid *et al.* (1991) who recorded inverse relationship of *Aeromonas* density with temperature of water. Further study is needed to explain the discrepancy between the findings of different investigators.

Different metallic ions may influence conductivity of water in the aquatic environment. Some of these metallic ions are used as enzymatic cofactors for growth, metabolic activity and survival of bacteria in aquatic ecosystem. Thus it is assumed that conductivity might play an important role in the survival of *Aeromonas* in the aquatic ecosystem. In the present study, significant correlation was found between

conductivity and *Aeromonas* density of water in both the areas (Tables-2.3 and 2.4). Our results are in partial agreement with the findings of Burke *et al.* (1984a).

Aeromonas is associated with the infection of fish (Davis 1953, Van Duijan 1967, Leblance *et al.* 1981) and other aquatic fauna (Shotts *et al.* 1972, Cussic and Bullock 1973, Larsen and Jansen 1977, Gorden *et al.* 1979). However, still it is not clear as to the concentration of *Aeromonas* spp. required to initiate disease manifestation in fish and aquatic animals in natural condition. In this study, the average density of *Aeromonas* in wastewater ranged between $4.0x10^1$ and $3.16x10^1$ and in non-wastewater areas ranged between $3.16x10^2$ and $2.50x\ 10^2$. But there was no *Aeromonas*-associated infection of fish and persons handling fish and duckweed. Therefore, further study is needed to ascertain the concentration (cfu/ml) of *Aeromonas* spp. and the favorable environmental condition necessary to initiate natural infection of fish by *Aeromonas* spp.

It may be concluded that the highest density of *Aeromonas* was recorded only in untreated wastewater. Hemolytic activity was recorded more among *A. sobria* than among *A. hydrophila* and *A. caviae*. Of the various kinds of sample, hemolytic activity was less in isolates from DW. Physicochemical parameters of water may influence the survival of aeromonads in the aquatic ecosystem. Moreover, no significant difference between wastewater and non-wastewater was observed in terms of *Aeromonas* counts in DW, water and fish samples of either area except untreated wastewater. Therefore, DW can be cultivated for treatment of wastewater and the same DW can also be used as fish-feed without adversely affecting pisciculture and persons handling DW and fish. These observations suggest that, duckweeds can be used as safe biological agent for wastewater treatment. Simultaneously the same DW can be used as fish-feed.

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CHAPTER – 3

AEROMONAS HYDROPHILA PHAGES IN SEWAGE-CONTAMINATED AND SEWAGE-FREE PONDS OF DHAKA CITY

3. *AEROMONAS HYDROPHILA* PHAGES IN SEWAGE-CONTAMINATED AND SEWAGE-FREE PONDS OF DHAKA CITY

3.1. INTRODUCTION

Aeromonas spp. are environmentally adopted microorganisms. Their cosmopolitan distribution in the aquatic ecosystem indicates that *Aeromonas* phages are also available in different ecological niches. Literature relating to phages of *Aeromonas* is less available than those on isolation of *Aeromonas*. Seeley and Primrose (1980) isolated *Aeromonas* phages for the first-time from the environment. They used this phage to conduct an ecological study of the effect of temperature on bacteriophage in an aquatic environment. Subsequently two morphologically similar *Aeromonas*-specific phages were isolated from the final effluent of a sewage treatment plant (Chow and Rouf 1983) of United States of America. One of these phages could infect 13 of 22 *A. hydrophila* strains while the other could infect only the host strain. This study indicated host specificity of two morphologically similar *Aeromonas* phages (Chow and Rouf 1983).

Aeromonas-specific bacteriophage PM_2 was isolated from freshwater samples of Spain (Merino *et al.* 1990a). This phage had a polyhedral head and a contractile tail. A low molecular weight lypopolysaccharide (LPS) fraction (LPS-core oligosaccharide) was identified as the only receptor for this phage. PM3 is another *Aeromonas*-specific phage (Merrino *et al.* 1990b), which specifically infected *A. hydrophila* strain TF7. An electron microscopic study revealed that

PM3 was a filamentous phage and the monopolar flagellum of the host *A. hydrophila* was the only receptor (Merino *et al.* 1990c). From Japan, Fukuyama *et al.* (1991) isolated *Aeromonas* phages from water and mud of a river to establish a phage-typing scheme of *Aeromonas*. By cross-matching test, isolated phages were classified into 13 types. These phages were able to type 21% (129 of 594) *Aeromonas* strains isolated from different aquatic ecosystems (Fukuyama *et al.* 1991) and from 51% of human diarrheal isolates of *Aeromonas* spp. collected from four different metropolitan hospitals of Tokyo (Fukuyama *et al.* 1992).

Ackermann *et al.* (1985) studied 34 *Aeromonas* phages. Electron microscopic examination revealed that the majority of the phages had contractile tails. These phages were classified into two families (*Myoviridae* and *Podoviridae*) and nine species. These phages resembled well-known phages of *Enterobacteriaceae,* such as T_2, P_1, P2 and T_7. Heads of these phages resembled T-even phage. Finally, it was concluded that these phages might have phylogenetic relationship with those of *Enterobacteriaceae* and T-even phages from morphological point of view. Moreover, Matsuzaki *et al.* (1999) reported morphologically similar *Aeromonas* (Aen 1 and 65), *Vibrio* (KVP 20, KVP40 and nt-1) and coli phage (T4). These three phages (aeromonad phages, *Vibrio* phages and coli phage T4) are distantly related to each other as evident from the N-terminal amino acid sequence analysis of major capsid protein (Matsuzaki *et al.* 1999).

In Bangladesh, strains of *Aeromonass* spp. have been isolated from pond (Islam *et al.* 1992), river and lake (Parveen *et al.* 1995), aquatic plants (Rahim *et*

al. 1985), ulcerated fish (Rahim *et al* 1984), prawns etc. (Rahim and Aziz 1988, Rahim and Aziz 1994). But isolation of *Aeromonas* phages has not been reported from Bangladesh. Therefore, this study was undertaken to assess the abundance of *Aeromonas* phages in water and sediment samples of sewage conta- minated and uncontaminated pond samples of Dhaka city.

3.2. MATERIALS AND METHODS

3.2.1. Collection of sample: Two water and two sediment samples were collected from sewage-free pond of the Institute of Public Health, Mohakhali, Dhaka. Five water and five sediment samples were collected from a sewage-contaminated pond of the same locality of Dhaka city. Water samples were collected in a 500 ml presterilized Nalgene plastic bottle and the sediment samples were collected in 4-ounce pre-sterilized glass-bottle. Samples were immediately transported to the laboratory in an insulating foam box without ice. Processing of the samples was completed within four hours of collection (Greenberg *et al.* 1992).

3.2.2. *Aeromonas* strains used: Ten local isolates of *Aeromonas hydrophila* (two strains isolated from human diarrheal stool and eight from ulcerated fish) were used in this study. Two reference strains of *Aeromonas* (*A. hydrophila* ATCC 7699 and *A. salmonicida* ATCC 14174) were also included in this study.

3.2.3. Screening of water and sediment samples for *Aeromonas* phages: One hundred microliters of overnight culture (10^{10}) of above-mentioned isolates were mixed with 100 µl of test water and sediment sample in 3 ml molten soft agar

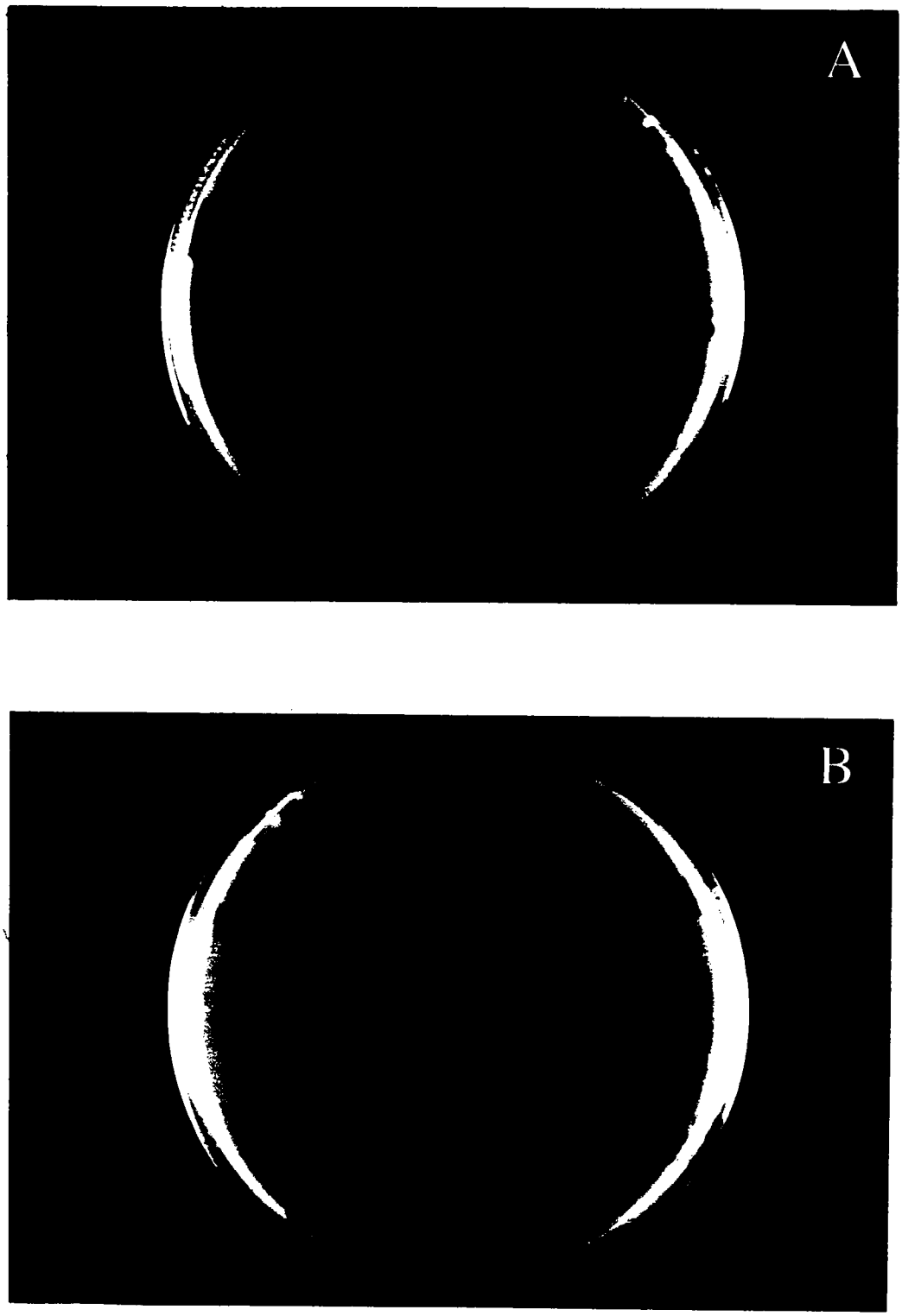

Fig. 3.1. Photographs showing plaque formation. Panel A shows complete lysis of plaque indicated by an arrow. Panel B shows incomplete lysis of plaque indicated by an arrow

(0.7%) in a screw cap tube. The sample was vortexed vigorously before pouring onto a Luria-Bartani (LB) agar plate. The inoculated plates were left on the table at room temperature until the top agar solidified. Then the plates were incubated at 37°C overnight. Following incubation, characteristic plaque formation was noted in the inoculated plate.

4.2.4. Lysis pattern: Morphologically different plaques were separately picked up. One well-isolated plaque and hundred microliters of overnight culture were mixed and kept at static condition at 37°C for one hour. Following incubation, mixture of host bacteria and the phage were inoculated into a 10 ml LB broth in a screw cap tube and incubated overnight in a shaking incubator at 37°C. Following incubation, one ml culture was centrifuged in eppendorf centrifuge (13,000 rpm for 10 minutes). The supernatant was taken in another tube to which chloroform was added to a final concentration of 1%. This mixture was vortexed vigorously and kept at 4°C as stock phage. From such preparation, 2 µl phage sample was spotted on LB agar plate with a top agar layer containing young culture of different *Aeromonas* strains used in this study. The inoculated plates were incubated overnight at 37°C. Following incubation, lytic patterns of phages were studied and graded "4+" when highly lytic and "-" when non-lytic. Fig. 3.1. shows (pannel A and B) shows lytic patterns.

3.3. RESULTS AND DISCUSSION

In the uncontaminated pond ecosystem, concentration of phages ranged between $6.0x10^1$ and $2.0x10^4$ pfu per ml or g of sample (Table-3.1). Phages were concentrated more in water column than in sediment. In sewage-contaminated pond samples, concentration of phages ranged between $4.0x10^1$ and $2.0x10^3$ pfu/ml or g of sample (Table-3.1). In sewage-contaminated samples, concentration of phages was higher in sediment than in water.

Aeromonas is considered an autochthonous flora of aquatic environment. Since it is not the normal flora of the gut, its abundance must be higher in the natural ecosystem than in sewage. Possibly for this reason *Aeromonas* phages were more abundant in the uncontaminated pond ecosystem than in sewage-contaminated one.

Enteric pathogens and indicators of fecal pollution undergo sedimentation from the water column after entering into an aquatic ecosystem (Grimes 1980). These bacteria persist in higher numbers in bottom sediment than in the water column. Bottom deposited sediment-bound bacteria are released into the water column when the sediment is disrupted mechanically (Grimes 1980). Similarly in the pond, possibly, as a result of bathing and swimming, sediment-bound *Aeromonas* phages are released into the water column. For this reason, concentration of *Aeromonas*-phages became higher in the water column compared to the sediment samples of the pond ecosystem. On the other hand, sewage-contaminated pond had no human interaction and sediment was not

Table-3.1. Concentration of *Aeromonas* phages in the pond and sewage samples of Dhaka eity

Source	*Aeromonas hydrophila* strains used											
	1	2	3	4	5	6	7	8	9	10	Ah	As
Pond 1 (W)[a]	0[d]	0	0	0	0	0	0	0	0	0	0	0
(S)[b]	0	0	0	0	0	0	0	0	0	0	0	0
Pond 2 (W)	0	0	$2x10^{3c}$	0	0	$1x10^4$	$2x10^4$	$1x10^4$	$1x10^4$	$1x10^3$	0	0
(S)	0	$6x10^1$	0	0	0	0	0	0	0	$6x10^1$	0	0
Sewage 1 (W)	0	0	0	0	0	0	0	0	0	0	$4x10^1$	0
(S)	0	0	0	0	0	0	0	$2x10^3$	0	$1x10^3$	$1x10^2$	0
Sewage 2 (W)	0	0	0	0	0	0	0	0	0	0	0	0
(S)	$2x10^2$	0	0	0	0	0	0	0	0	0	0	0
Sewage 3 (W)	0	0	0	0	0	0	0	0	0	0	0	0
(S)	0	0	0	0	0	0	0	0	0	0	0	0
Sewage 4 (W)	0	0	0	0	0	0	0	0	0	0	0	0
(S)	0	0	0	0	0	0	0	0	0	0	0	0
Sewage 5 (W)	0	0	0	0	0	0	0	0	0	0	0	0
(S)	0	0	0	0	0	0	0	0	$4x10^1$	0	0	0

[a] Water, [b]Sadiment, [c]Less than 10 pfu per g or ml sample, [d] pfu per g or ml of sample, Ah=*A. hydrophila* ATCC 7699, As= *A. salmonicida* ATCC 14174

Table-3.2. Lysis pattern of various *Aeromonas hydrophila* strains with the *Aeromonas* phages

	A. hydrophila phages																		
A. hydrophila	7	8	9	10	11	12	14	15	17	19	20	21	22	23	24	25	26	27	28
1	++++	+	++++	++++	++++	-	++++	++++	-	++++	-	-	+++	++++	++++	++++	++++	-	+
2	++++	++	++++	++++	++++	-	++++	++++	-	++++	++++	++++	-	++++	-	-	++++	++	-
3*	++++	+	++++	++++	++++	-	++++	++++	-	++++	++++	++++	-	++++	-	-	+++	++++	-
4*	-	-	-	-	-	-	-	-	-	-	++++	++++	-	-	-	-	-	++++	-
5*	++++	+	++++	++++	+	-	++++	++++	-	++++	-	-	-	++++	-	-	-	-	-
6	++++	+++	++++	++++	++++	-	++++	++++	-	++++	++++	++++	-	++++	-	-	++++	-	-
7	++++	+++	++++	++++	++++	++++	++++	++++	-	++++	++++	++++	-	++++	-	++++	++++	-	+
8	++++	+	++++	++++	++++	-	++++	++++	++++	++++	++++	++++	-	++++	++++	++++	++++	++++	+
9	++++	++++	+++++	++++	++++	++++	++++	++++	-	++++	++++	++++	-	++++	-	-	++++	-	-
10	++++	+	++++	+++	+++	++++	+++	++++	-	++	+++	++++	-	-	++++	++++	++++	++++	+
Ah	+	++++	++++	++++	+++	++++	+++	++++	-	++	+++	++++	-	-	++++	++++	++++	++++	+
As	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Enterotoxigenic strain; [a]strain 1 and 2 were isolated from human and No. 3-10 were from ulcerated fish; [b]strain of *A. hydrophila*; Ah=*A. hydrophila* ATCC7966[b]; As = ATCC14174[c] [a]a strain of *A.salmonicida*[c]; degree of lysis= (++++) (highest);- (no lysis), phage 1-6 degenerated and could not be studied

disturbed. As a result normal phenomenon went on here. Thus, *Aeromonas* phages remained concentrated in sediment than in water column in sewage-contaminated pond (Table-3.1).

Bacteriophages show host-specificity with respect to a particular bacterium or same bacteria of different serotype. Based on this principle, different phage-typing schemes of bacteria have been proposed, such as phage-typing scheme of *V. cholerae* O1 (Chattopadhyay *et al.* 1993). Host specificity was also demonstrated by different phages of *Aeromonas* (Merino *et al* 1990b). In this study, these phages showed variable sensitivity patterns with respect to different strains of *A. hydrophila* but failed to infect *A. salmonicida* (ATCC 14174) (Table-3.2). Therefore, these phages are specific for *A. hydrophila*.

Based on the findings of this study, it may be concluded that *A. hydrophila* phages are distributed in sewage-contaminated and uncontaminated pond samples of Bangladesh. Concentrations of *Aeromonas* phages are more in uncontaminated than in sewage-contaminated samples. Further work is needed to constitute a phage-typing scheme for rapid identification and typing of *A. hydrophila* strains.

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CHAPTER – 4

EVALUATION OF PATHOGENIC POTENTIAL OF CYTOTOXIC ENTEROTOXIN GENE-POSITIVE ISOLATES OF *AEROMONAS* SPP.

4. EVALUATION OF PATHOGENIC POTENTIAL OF CYTOTOXIC ENTEROTOXIN GENE-POSITIVE ISOLATES OF *AEROMONAS* SPP.

4.1. INTRODUCTION

Aeromonas spp. are associated with life-threatening diseases of humans, such as bacteremia (Ko *et al.* 2000), meningitis (Lin and Cheng 1998), septicemia (Tabata *et al.* 1999), myonecrosis (Balasco *et al.* 1995), lung abscess (Hur *et al.* 1995), pulmonary infection (Lecler *et al.* 1990) etc. It is one of the important agents associated with diarrhea (Kuijper *et al.* 1991). *Aeromonas* spp. associated enteric infection is common among young children manifesting diarrheal symptom (Teka *et al.* 1999) and are also associated with travellers' diarrhea (Yamada *et al.* 1997). Recently, strains of *Aeromonas* spp. have been incriminated as one of the agents associated with hemolytic-uremic syndrome (Robson *et al.* 1992, Fang *et al.* 1999). Prawns infested with *Aeromonas* spp. can multiply when stored at 4°C (Rahim and Aziz 1994) and thus these bacteria may be responsible for spoilage of food stored at lower temperature. *Aeromonas* spp. have been identified as one of the important food-borne pathogens (Janda *et al.* 1999). Infestation of *Aeromonas* spp. in ready-to-eat food, such as salad, is considered a risk factor from public health point of view (Mattick and Donovan 1998).

Aeromonas spp. can produce different virulence associated putative factors, such as straight (Sato *et al.* 1989) and flexible pili (Ho *et al.* 1990). These pili are of class IV type. Two distinct class IV type pili (bundle forming and Tap) are produced by *Aeromonas* spp. (Kirov *et al.* 1999, Kirov *et al.* 2000) and are

associated with virulence. Other putative virulence-associated factors of *Aeromonas* spp. are collagen-binding protein (Gullberg *et al.* 1989), hemagglutinin (Majeed and Macrae 1994) etc. Besides, *Aeromonas* spp. can produce S-layer (Kokka *et al.* 1991). Strains having S-layer can resist bactericidal activity of 65% of pooled human serum (Janda *et al.* 1994). These strains are moderately pathogenic to mice than S-layer negative strain (Janda *et al.* 1994). Moreover, *Aeromonas* strains of serotype 0:34 can resist complement-mediated lysis (Merino *et al.* 1994). These properties help the bacteria to cause bacteremia (Ko *et al.* 2000) and septicemia (Tabata *et al.* 1999). Through blood stream, invading strains of *Aeromonas* spp. can be disseminated to other parts of the body to infect vital organs, such as lung (Hur *et al.* 1995), heart etc. (Blasco *et al.* 1995).

Strains of *Aeromonas* spp. produce several virulence factors associated with enteric infections, such as enterotoxin (Chopra and Houston 1999), cytotoxin (Alavandi *et al.* 1999) and hemolysin (Fujii *et al.* 1999). Chakraborty *et al.* (1986) cloned cytotoxin gene of *A. hydrophila* and conclusively proved its role in pathogenesis of enteric infection. Hemolytic, cytotoxic and enterotoxin gene (*act*) of a diarrheal isolate, *A. hydrophila* SSU, has been cloned (Chopra *et al.* 1993). This gene codes for a toxin protein (Act), which is of 52 kDa in size. This protein is lethal in mice when injected intravenously (Rose *et al.* 1989). Subsequently, multiple biological functions of different loci of a single peptide chain (Act) have been confirmed by site directed mutagenesis and preparation of anti-peptide antibody (Ferguson *et al.* 1995). In the present study, 69 isolates of *Aeromonas* spp. were screened for the presence of cytotoxic enterotoxin (*act*)

gene. Furthermore, pathogenic potential and drug resistance patterns of *act* probe-positive and probe-negative isolates were also tested in this study.

4.2. MATERIALS AND METHODS

4.2.1. Bacterial strains: Sixty-nine isolates of *Aeromonas* spp. (*A. hydrophila* n=22, *A. sobria* n=41 and *A. caviae* n=6) were used in this study. These strains were isolated from water, duckweed (*Lamna minor*) and fish during August 1994 through July 1995.

4.2.2. Extraction of DNA: *Aeromonas* strains were retrieved from stock-culture on Luria-Bertani (LB) agar plate at 37°C. Portion of one well-isolated aeromonad colony was inoculated into 5 ml LB broth and incubated overnight at 37°C in a shaking incubator. From 1 ml overnight culture, chromosomal DNA was extracted and purified using WIZARD genomic DNA purification kit (California, USA). Pelleted DNA was dissolved in 100 µl of DNA dissolving solution of the kit (WIZARD genomic DNA purification kit, California, USA). Then the chromosomal DNA was diluted in the proportion of 1:50 and the concentration of DNA was measured at OD_{260} using UV-1201 spectrophotometer (P/N 206-62409 model, Shimadzu Corporation, Tokyo, Japan). DNA was further diluted in deionized sterilized distilled water to a final concentration of 0.1 µg/µl.

4.2.3. Dot blotting: 10 µl DNA of solution (1 µg) was taken from the stock DNA (0.1 µg/ul) prepared as mentioned above in a PCR tube. To this, 90 µl of final concentration equal to 0.4 M NaOH and 10 mM EDTA were added, heated at 94°C for 10 minutes and immediately chilled in ice for 10 minutes. 100 µl 2M-

ammonium acetate was mixed with denatured chilled DNA for dot blotting.

A piece of Zeta Probe membrane (BIO-RAD) was taken from the packet with the help of a pair of forceps and soaked in de-ionized sterilized distilled water for 10 minutes. For dot blotting, microfiltration apparatus was assembled with a piece of pre-wetted Zeta Probe membrane (BIO-RAD) as per instructions of the manufacturer and 500 µl de-ionized distilled water was passed through each well. Then 200 µl of DNA samples (as prepared above) was passed through each well, which was further rinsed with 500 µl of 0.4M NaOH. The microfiltration apparatus was disassembled to take out the Zeta Probe membrane with immobilized DNA and rinse in 2xSSC. Finally, UV-cross linking of DNA with the Zeta Probe membrane was performed with the GS Gene Linker UV chamber. The membrane was then stored dry between two pieces of filter paper in a plastic bag at room temperature.

4.2.4. Preparation of probe

4.2.4.1. Probe: *A. hydrophila* SSU cytotoxic enterotoxin (*act*) probe was a 0.6 kb DNA fragment (Chopra *et al.* 1993). For labeling, non-radio active ECL direct nucleic acid labeling and detection system (Amersham Life Science, U.K.) was used. The detailed procedure was as follows: The initial concentration of probe was 20 µg/µl. 5 µl (100 µg) of unlabelled DNA fragment corresponding to *act* gene probe was mixed with 5 µl of sterilized deionized distilled water, boiled for 5 minutes and chilled immediately in ice. 10 µl of labeling reagent (Amersham Life Science, UK) was mixed with the denatured DNA fragment and incubated at

37°C for 20 minutes. Following incubation, the mixture was thoroughly mixed, centrifuged briefly and kept on ice for using this probe immediately.

4.2.5. Pre-hybridization: Zeta Probe membrane containing immobilized DNA of different *Aeromonas* isolates was taken in 30 ml of hybridization solution (30 ml Gold hybridization buffer, 1.5 g blocking agent, 0.876 g NaCL) in a suitable leak-proof hybridization bag. Air bubble was completely removed from the hybridization bag, sealed and immersed in water for 2 hours in a water bath at 42°C with gentle agitation.

4.2.6. Hybridization: Following incubation, 20 µl of *act* probe (as prepared above) was added in the hybridization solution containing blot, mixed thoroughly and bubbles removed completely. Finally, the bag was again sealed for overnight incubation at 42°C in a water bath with gentle agitation.

4.2.7. Post-hybridization washing: Following hybridization, the blot was washed in 500 ml of primary wash buffer (0.5xSSC, 0.4% SDS) twice at 55°C for 20 minutes each with gentle agitation and twice in secondary wash buffer (2x SSC) at room temperature for 5 minutes each with gentle agitation. Then the blot was taken on a clean container in a moist condition for generation of signal.

4.2.8. Signal generation on X-ray film: Five milliliter each of detection reagent 1 and 2 were added on moist hybridized blot with DNA side up for 1 minute. Excess detection reagent was drained out from the blot, which was covered with saran wrap and fixed inside one X-ray film cassette rapidly. Then this blot was

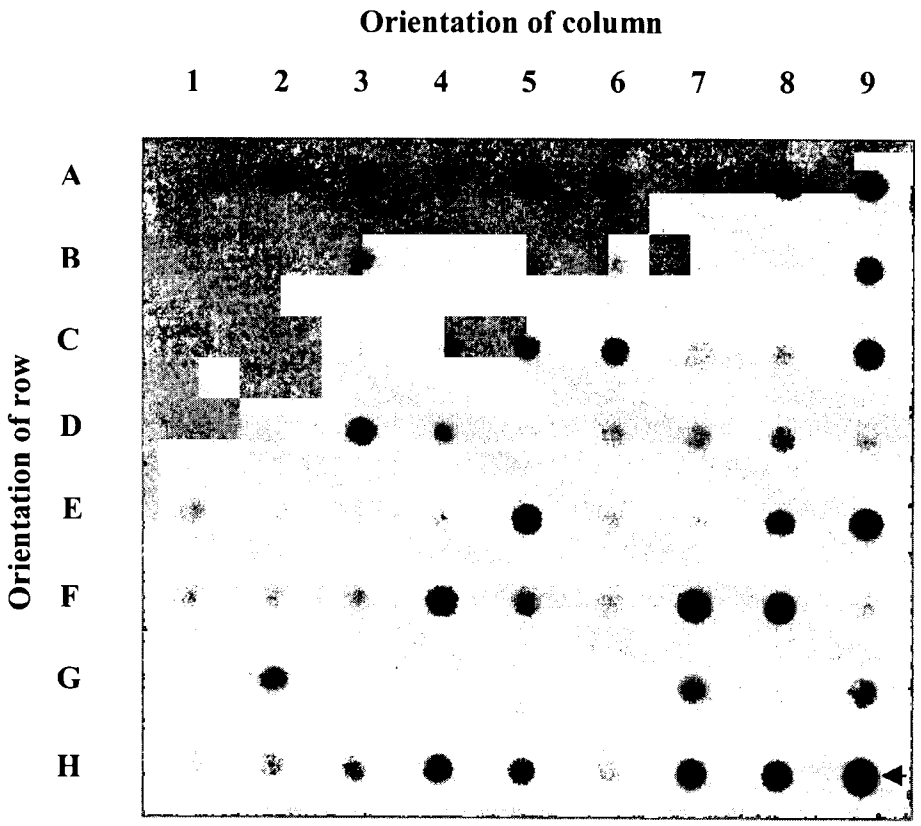

Fig. 4.1. X-ray film exposed to hybridized Zeta Probe membrane showing signals of hybridization. Arrow indicates positive control (orientation H 9)

exposed to X-ray film in dark room at least for 5 minutes at ambient temperature before developing the film for generation of prominent signal of hybridization.

4.2.9. Preparation of culture filtrate (CF): Isolates of *Aeromons* spp. harboring *act* gene were retrieved on gelatin agar plate. Portion of one well isolated young colony of *Aeromonas* was inoculated into 5 ml T1N1 [(1% trypticase (Difco laboratories), 1% NaCL (Fisher Scientific) pH-7.2)] and incubated overnight at 37°C in a shaking water bath (120 rpm/min). Following incubation, 500 µl overnight culture was inoculated in 10 ml Richardson's medium (Richardson 1969) in 50 ml conical flask and incubated overnight at 37°C in a shaking water bath (120 rpm/min). Then the culture was centrifuged in RC5C centrifuge (RC5C Sorvall Instrument, DU PONT, USA) at 10,000 rpm for 15 min at 4°C. Following centrifugation, supernatant was decanted and further sterilized by syringe filter (0.22 µm pore, Gelman Science, USA). Cell-free CF samples were aliquoted in 1.5 ml eppendorf centrifuge tubes and stored at -20°C until use. CF was also prepared from *Vibrio cholerae* 569B (positive control in RIL assay), *E. coli* 36004 (positive control in suckling mice assay) and *E. coli* 36000 (negative control in suckling mice assay) following the same procedure and stored similarly.

4.2.10. Heat inactivation of culture filtrate (CF): CF aliquots were completely defrosted at room temperature. Two aliquots prepared from same strain were then heated separately at 56°C and 100°C for 20 minutes each.

4.2.11. Suckling mice assay: Suckling mice assay was performed following the

procedure of Dean *et al.* (1972). In short; a group of three-day old mice (Hanover strain, NMRI) were separated from their mothers. 0.1ml CF containing 0.01% (wt/vol) Evans blue was directly injected into the milky-white stomach of two of three suckling mice and the third, without injection of CF, was used as negative control. After three hours of incubation at room temperature (22°C), these mice were killed by cervical dislocation. Their intestines were removed, pooled and weighed. Fluid accumulation was expressed as the ratio of weight of the intestine to the remaining body weight. A ratio ≥0.08 was considered positive whereas ratio less than this was considered negative. CF prepared from *E. coli* 36004 and *E. coli* 36000 was similarly inoculated as positive and negative controls, respectively.

4.2.12. Rabbit ileal loop (RIL) assay: Enterotoxicity of the isolates of *Aeromonas* spp. harboring *act* gene was tested in RIL following the procedure of Sanyal *et al.* (1975). Briefly, eight samples were tested in eight separate loops each with an interloop between two consecutive loops of a rabbit (New Zealand white variety weighing 1.2 to 1.9 kg). One milliliter of CF was injected in each loop of a rabbit ileum in duplicate rabbit following laparotomy. In the first loop, 1 ml CF of *V. cholerae* 569B was injected as positive control and 1 ml sterile normal saline was injected in the eighth loop as negative control. In the remaining loops between positive and negative controls, 1 ml CF of six test isolates was inoculated sequentially. The inoculated rabbit was sacrificed after 16-18 hours of incubation to measure the volume of fluid accumulation per cm of gut in each rabbit. Fluid accumulation equal or more than 0.5 ml/cm of gut was considered positive reaction in RIL.

4.2.13. Vero cell assay: Cytotoxic effect of the culture filtrate of *Aeromonas* spp. harboring *act* gene was tested in Vero cell-line (African green monkey kidney cells). Vero cells were grown in Eagle's Minimal Essential Medium (EMEM) supplemented with L-glutamate (2.3 mM), heat-inactivated fetal bovine serum (10%) (GIBCO BRL), penicillin (100000 U/L) and streptomycin (10 mg/L). Two hundred microlitres of Vero cells suspended in EMEM ($2x10^3$/ 200 µl) were placed in wells of microtitre plate (96 well, 15 cm^2, flat bottom microtiter plates) (Falcon) and incubated at 37°C in a humidified Co_2 incubator for three hours for adhesion. Serial two-fold dilutions of CF filtrate were prepared in PBS and 200 µl of crude toxin sample was added into each well of the microtiter plate containing freshly prepared cell. A plate containing Vero cell and toxin was incubated at 37°C in humidified Co_2 incubator and change of the cell morphology (cell death or rounding) was studied using an inverted microscope after 18-24 hours of incubation.

4.2.14. Test of hemolytic activity: Hemolytic activity of *act* gene-positive *Aeromonas* isolates was tested on blood agar plate containing 5% defibrinated sheep blood. Initially, the strains were subcultured onto gelatin agar (GA) plate and incubated at 37°C overnight. Lower surface of the blood agar plate was marked into several squares of 0.7 cm each and marked with test strain number. Portion of colony from the overnight *Aeromonas* culture from the GA plate was transferred into one chamber having a corresponding isolate number. The

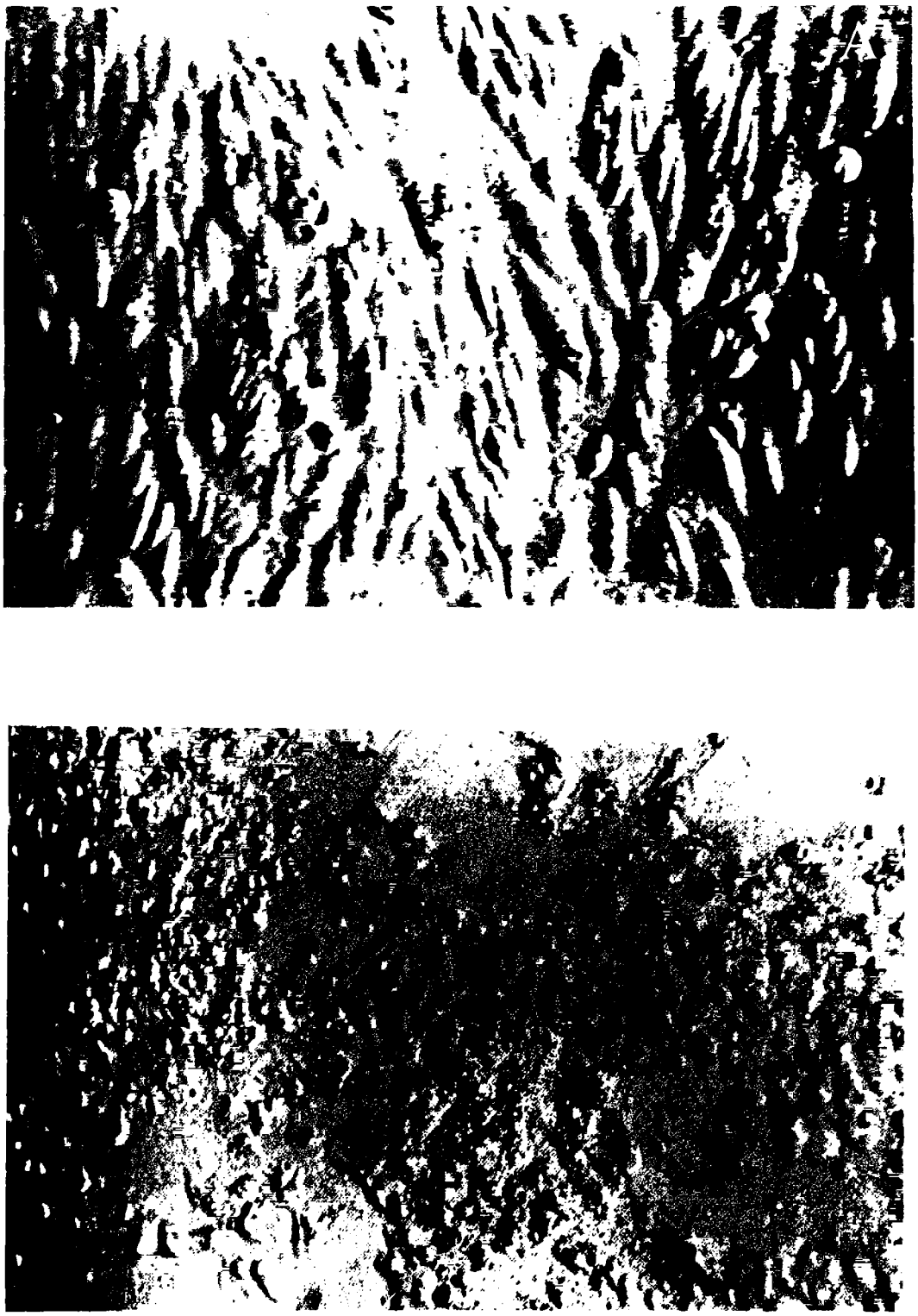

Fig. 4.2. Vero cell assay. Panel A shows normal Vero cell and panel B shows toxic effect on Vero cell

inoculated plates were incubated aerobically at 37°C overnight and at microaerophilic condition using a candle jar. Following incubation, hemolytic strains showed prominent zone of hemolysis around each colony were characterized α, β and γ.

4.2.15. Test of CAMP-hemolysin: Overnight culture of *Aeromonas* spp. harboring *act* gene and *Staphylococcus aureus* (ATCC 25923) were inoculated 8 mm apart on blood agar plate containing 5% washed sheep blood plate. Inoculatd plates were incubated at 37°C in candle jar for 18-24 hours (Lesmana *et al.* 1994). Following incubation, CAMP-positive isolates of *Aeromonas* could only produce characteristic type of hemolytic activity between test *Aeromonas* isolate and *S. aureus* ATCC 25923.

4.2.16. Test of drug susceptibility: Antibiotic sensitivity of *Aeromonas* spp. harboring *act* gene and *act* gene-deficient isolates was performed following the single disc agar diffususion method of Bauer *et al.* (1966). These disks were mostly purchased from MAST diagnostics (MAST group, Merseyside, UK) except otherwise indicated. The antibiotics and chemotherapeutic agents used were (in microgram): ampicillin 10, chloramphenicol 30 (Oxoid, UK), ciprofloxacine 5, co-trimoxazole 25, erythromycin 15, furazolidone 100 (OXOID, UK) and tetracycline 30.

4.2.17. Data analysis: Chi-square test was performed using SPSS10 to find out the difference between *act* gene-positive and gene-negative isolates of *Aeromonas* spp. with respect to different bioassays.

4.3. RESULTS

4.3.1. Probe assay: Prominent signal of hybridization was obtained from 32 of 69 different *Aeromonas* DNA dotted on the Zeta Probe membrane. X-ray film exposed to hybridized Zeta Probe membrane is shown in Fig. 4.1.

4.3.2. Enterotoxicity in suckling mice assay (SMA): Ten CF of 32 *act* gene-positive and 2 of 31 *act* gene-negative isolates of *Aeromonas* spp. induced fluid accumulation in SMA (Table-4.1). Strains of *Aeromonas* spp. harboring *act* gene differed from *act* gene-deficient strains with respect to enterotoxicity in SMA. This difference was significant (P=0.011) (Table-4.2).

4.3.3. Vero cell assay: CF of 31 *act* gene-positive and 30 gene-negative isolates of *Aeromonas* spp. were tested for cytotoxicity in Vero cell-line. Fig. 4.2 (pannel B) shows morphological change of Vero cell in response of cytotoxic enterotoxin of *Aeromonas* spp. Twenty-five of 31 *act* gene-positive and 27 of 30 *act* gene-negative isolates of *Aeromonas* spp. showed cytotoxicity in Vero cell-line. Cytotoxicity of *act* gene-positive isolates of *Aeromonas* spp. could not be differentiated from *act* gene-negative ones with respect to Vero cell assay. This difference was not significant (P=0.473) (Table- 4.2).

4.3.4. Rabbit ileal loop (RIL) assay: Twenty-six CF of *Aeromonas* spp. harboring *act* gene were tested in RIL. Three of 26 CF could induce fluid accumulation in RIL assay. Fluid accumulation ranged between 0.9 and 1.3 ml/cm of gut (Table 4.1).

Table-4.1. Hemolytic activity and toxigenic potential of *act* gene-positive and gene-negative isolates of *Aeromonas* spp. in animal model and cell-line

Characteristics of strains (n)	Suckling mice assay with culture filtrate		Vero-cell assay with culture filtrate		CAMP-hemolysin assay	Rabbit ileal loop assay
	Unheated (sample positive/ tested)	Heated at 56°C (sample positive/ tested)	Unheated (sample positive/ tested)	Heated at 56°C (sample positive/ tested)	(sample positive/ tested)	(sample positive/ tested)
act probe positive isolates (n=32)	10/30	0/30	25/31	0/31	14/32	3/26
act probe positive isolates (n=31)	2/31	ND	27/30	ND	8/31	ND*

*ND= not done

Table-4.2. Difference between *act* gene-positive and gene-negative isolates of *Aeromonas* spp. with respect to different bioassays

Bioassays	*act* gene positive isolates No. positive/ No. tested	*act* gene negative isolates No. positive/ No. tested	Exact significance (P value)
Suckling mice assay	10/30	2/31	0.011
Vero cell assay	25/31	27/30	0.473
Hemolysin assay	30/31	22/31	0.012
CAMP-hemolysin	14/32	8/30	0.192

Table-4.3. Drug sensitivity of *act* gene-positive and gene-negative *Aeromonas* isolates

Antibiotics/ Chemotherapeutic agents	Type of response of	
	***act* gene-positive *Aeromonas* spp. (n=24)**	***act* gene-negative *Aeromonas* spp. (n=24)**
	Sensitive (%)	**Sensitive (%)**
Ampicillin	10(41.7)	4(16.7)
Chloramphenicol	24(100)	24(100)
Ciprofloxacin	24(100)	24(100)
Co-trimoxazole	22(91.7)	21(87.5)
Erythromycin	1(4.2)	0
Furazolidone	24(100)	24(100)
Tetracycline	23(95.8)	24(100)

4.3.5. Hemolytic activity: Hemolytic activity (both α and β) of both *act* gene-positive and gene-negative isolates were tested at aerobic condition of overnight incubation at 37°C on blood agar plate (Table-4.1). Hemolytic activity (both α and β)) was detected in 30 of 32 *act* gene-positive and 22 of 30 *act* gene-negative isolates of *Aeromonas* spp. Isolates of *Aeromonas* spp. harboring *act* gene differed from *act* gene-deficient ones with respect to hemolytic activity on blood agar plate. This difference was significant (P=0.012) (Table-4.2).

Twenty-six *act* gene-positive isolates were incubated overnight at 37°C at microaerophilic condition (candle jar) to study the effect of oxygen on hemolytic activity during incubation. Of them, 23 showed hemolytic activity (Table-4.1). Three isolates (Nos. 97, 155 and 187) produced β-hemolytic activity aerobically but these isolates produced α-hemolytic activity at microaerophilic condition. Moreover, isolate 26 showed α-hemolytic activity at aerobic condition but this isolate failed to produce hemolytic activity at microaerophilic condition. From this result, it can be concluded that hemolytic activity of *Aeromonas* spp. may be enhanced due to incubation of isolates at aerobic condition.

4.3.6. CAMP-hemolysin: Production of CAM-hemolysin was detected in 14 of 32 *act* gene-positive and 8 of 30 *act* gene-negative *Aeromonas* isolates. Some of the isolates produced crescent shaped CAMP-hemolytic zone while others produced prominent β-hemolytic zone extended towards growth of *S. aureus*. Strains of *Aeromonas* spp. harboring *act* gene could not be differentiated from *act* gene-deficient isolates with respect to CAMP-like hemolysin test (P=0.192) (Table-4.2).

4.3.7. Drug resistance pattern: Twenty four randomly selected *Aeromonas* spp. harboring *act* gene and an equal number of *act* gene-negative *Aeromonas* isolates were tested for sensitivity to commonly used antibiotics and chemo- therapeutic agents (Table-4.3). All *act* gene-positive isolates were uniformly resistant to erythromycin and sensitive to tetracycline. However, all *act* gene- positive and gene-negative isolates were uniformly sensitive to chloramphenicol, ciprofloxacin and furazolidon. Some of the isolates showed a variable sensitivity pattern with respect to other antibiotics (Table-4.3).

4.3. DISCUSSION

Suckling mice assay (SMA) was initially devised for evaluating production of heat-stable enterotoxin of *E. coli* (Dean *et al.* 1972). Considering this assay as relatively cheaper and faster, Burke *et al.* (1981) tested enterotoxicity of strains of *A. hydrophila* collected from different geographical areas and established SMA as the model of testing enterotoxicity. Subsequently, several investigators successfully used SMA for testing enterotoxicity of *Aeromonas* spp. (Haque *et al.* 1996, Wang *et al.* 1996). In this study, more *act* gene-positive isolates showed enterotoxic activity compared to gene-negative ones. Mice lethality by injecting Act has been tested (Ferguson *et al.* 1997) but SMA for testing enterotoxicity of Act was not evaluated. Therefore, from this study, it is evident that SMA assay could be used as one of the assays for testing enterotoxicity of Act.

Sanyal *et al.* (1975) first demonstrated enterotoxicity of live culture of clinical and environmental isolates of *A. hydrophila* in rabbit ileal loop model

(RIL). Using this model, Annapurna and Sanyal (1977) from India and Wadstrom *et al.* (1976) and Ljungh *et al.* (1981) from Sweden simultaneously demonstrated enterotoxicity of cell-free CF of *Aeromonas* isolated from clinical and environmental samples. Thus, RIL is an established assay for testing enterotoxicity of *Aeromonas*. RIL can be used for detecting *Aeromonas* cytotoxic enterotoxin (Act) in cell-free CF of *A. hydrophila* (Ferguson *et al.* 1997). It has been demonstrated that 200 ng is the minimum amount of Act required to induce fluid accumulation in RIL (Ferguson *et al.* 1997). In this study, CF of only three of 32 *act* gene-positive isolates could induce fluid accumulation in this assay. It is possibly due to the fact that gene-positive isolates other than these three produced Act less than 200 ng in Richardson's medium, which was not detected in RIL. To confirm this observation, it is essential to evaluate various culture media for the better production of Act.

Vero toxin, which is also called Shiga-like toxin, is one of the virulence factors in the pathogenesis of gastroenteritis. This toxin was initially detected in *Shigella dysenteriae* type 1 (Stockbine *et al.* 1988). Subsequently this toxin was reported mostly from various serotypes of *E. coli* (O'Brian *et al.* 1982, Cleary *et al.* 1985) and other bacteria (O'Brian *et al.* 1984). Vero toxin producing *Aeromonas* was reported from meat samples from Australia (Majeed and Macrae 1994) and from clinical and environmental samples from Japan (Haque *et al.* 1996). In this study, Vero toxin has been reported both from *act* gene-positive and gene-negative strains of *Aeromonas* spp. isolated from the environment. It indicates that both *act* gene-positive and gene-negative isolates of *Aeromonas* spp. tested in this study are pathogenic for humans. This is the first report of its

kind from Bangladesh.

Aeromonas spp. isolated from fish (Boulanger *et al.* 1977), clinical specimens and environmental samples (Burke *et al.* 1982, Singh and Sanyal 2000) could lyse red blood cells (RBC) of different species. On blood agar plate, *Aeromonas* spp. produce two different types of hemolysins, such as α and β (Ljungh *et al.* 1982). In this study, *Aeromonas* strains produced all two types of hemolysins. This observation is consistent with past reports from Bangladesh (Parveen *et al.* 1992). Moreover, hemolysin is one of the virulence factors of *Aeromonas* (Chakraborty *et al.* 1987). Purified hemolysin showed multiple biological properties, including hemolytic, cytotoxic and enterotoxic activities and lethality in mice (Asao *et al.* 1984) like Act of *Aeromonas* (Chopra *et al.* 1993). Although hemolytic activity is one of the biological properties of Act, molecular cloning and DNA sequence analysis of *act* gene from *A. hydrophila* (Chopra *et al.* 1993) revealed that this gene differed significantly from aerolysin gene of *A. trota* (Chakraborty *et al.* 1986) and hemolysin gene *A. hydrophila* reported by Asao *et al.* (1984). Due to this type of structural difference at the nucleotide sequence, *act* probe, which was used in this study, possibly failed to hybridize with other hemolytic isolates of *Aeromonas* spp.

Synergistic CAMP-hemolysin was introduced by Christie *et al.* (1944). This test has been successfully used for differentiating *V. cholerae* O1 El Tor (positive) from classical strains (negative) (Lesmana *et al.* 1994). In different species of *Aeromonas*, CAMP-hemolysin production differed at different atmospheric condition of growth, such as aerobic or microaerophilic condition

(Figura and Guglielmatti 1987, Altwegg *et al.* 1990). Based on CAMP-like test, Carnahan *et al.* (1991) differentiated *A. hydrophila* and *A. veronii* bv. *sobria* (both positive) from *A. caviae* (negative). Subsequently, Gubash (1997) conducted CAMP-hemolysin test of a huge number of *Aeromonas* isolates and raised question about the usefulness of CAMP-hemolysin to differentiate different strains of *Aeromonas* spp. However, none of these authors made any attempt to correlate pathogenicity of *Aeromonas* with CAMP-hemolysin. In the present study, some of the pathogenic properties of *Aeromonas* correlated with CAMP-hemolysin (Table-4.2). Thus, pathogenic factors could be easily assessed in fresh *Aeromonas* isolates by simply doing CAMP-hemolysin test. Therefore, pathogenic properties of *Aeromonas* could be easily tested economically in laboratories of developing countries where it is often difficult to set up experiments involving dot-blot hybridization, animal model and tissue culture assay for testing enterotoxicity.

Aeromonas has been reported to be uniformly resistant to ampicillin (Joseph *et al.* 1979) and ampicillin resistance was found to be uniform among *A. hydrophila* isolated from diarrheal patients in Bangladesh (Aziz *et al.* 1986). In the present study, sensitivity of *act* gene-positive and *act* gene-negative strains of *Aeromonas* to ampicillin was variable (Table-4.3). This observation is consistent to our previous study of *Aeromonas* isolated from infected fish (Rahim *et al.* 1984) and fresh water prawn samples (Rahim and Aziz 1994). Chloramphenicol is not used for the treatment of diarrhea in Bangladesh but some isolates of *Aeromonas* from patients with diarrhea were resistant to this antibiotic (Aziz *et al.* 1986). In the present study, both *act* gene-positive and

gene-negative isolates were uniformly sensitive to chloramphenicol. Ciprofloxacin and furazolidone are used in the treatment of diarrheal disease but *Aeromonas* isolates of this study were uniformly sensitive to these two antibiotics. Both *act* gene-positive and gene-negative isolates of *Aeromonas* spp. in this study showed variable sensitivity patterns with respect to co-trimoxazole. These results are consistent with previous reports from Bangladesh (Rahim *et al.* 1984, Rahim and Aziz 1994). Similar to our previous report (Rahim *et al.* 1984), *act* gene-positive and gene-negative strains of this study showed variable sensitivity patterns with respect to erythromycin but unlike previous study (Rahim and Aziz 1994), *act* gene-negative isolates were uniformly sensitive to this antibiotic. Consistent to the previous report from Bangladesh (Rahim *et al.* 1984, Rahim and Aziz 1994), both *act* gene-positive and gene-negative isolates showed variable sensitivity patterns with respect to tetracycline. Compared to our previous study (Rahim *et al.* 1984, Rahim and Aziz 1994), slight discrepancies of sensitivity pattern of *Aeromonas* isolates of this study were observed with respect to some antibiotics and chemotherapeutic agents. This phenomenon is possibly due to environmental variation and time difference of isolation.

It is evident from this study that environmental isolates of *Aeromonas* spp. tested in this study (32 of 69) harbor cytotoxic enterotoxin gene (*act*). Strains harboring *act* gene significantly differed from *act* gene-deficient strains with respect to enterotoxicity in suckling mice assay and hemolytic activity on blood agar plate. Moreover, compared to *Aeromonas* strains harboring *act* gene, gene-negative isolates were uniformly resistant to erythromycin and sensitive to

tetracycline. Both *act* gene-positive and gene-negative isolates showed variable sensitivity patterns with respect to different antibiotics tested.

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CHAPTER - 5

EFFECT OF CULTURE CONDITIONS ON PRODUCTION OF CYTOTOXIC ENTERO-TOXIN BY *AEROMONAS SOBRIA*

5. EFFECT OF CULTURE CONDITIONS ON PRODUCTION OF CYTOTOXIC ENTEROTOXIN BY *AEROMONAS SOBRIA*

5.1. INTRODUCTION

Aeromonas is one of the bacterial agents associated with enteric infections (Teka *et al.* 1999). It is also associated with life-threatening diseases of humans such as septicemia (Tabata *et al.* 1999, Ko *et al.* 2000), meningitis (Lin and Cheng 1998), corneal ulcer (Carta *et al.* 1994), pulmonary infection (Lecler *et al.* 1990) and hemolytic-uremic syndrome (Fang *et al.* 1999, Robson *et al.* 1992). *Aeromonas* spp. associated human wound infections have been reported due to exposure of divers in polluted water (Daily *et al.* 1981).

Aeromonas spp. are autochthonous flora of the aquatic environment. In aquatic ecosystems, density of aeromonads fluctuates with variations of physicochemical parameters of water (Fliermans *et al.* 1977). Under certain unknown physicochemical conditions of water, aeromonads acquire virulence and infect a variety of aquatic animals, including fish (Leblanc *et al.* 1981), alligators (Shotts *et al.* 1972), dolphins (Cusick and Bullock 1973) and snake (Heywood 1968). *A. hydrophila* can also infect domestic animals, such as dog (Andre-Fontaine I995) and cow (Wohlgemuth *et al.* 1972).

Aeromonas spp. produce different virulence factors (Sanyal *et al.* 1975, Wadstrom *et al.* 1976). Independent reports indicate the production of extra-cellular heat-labile enterotoxin of *Aeromonas* from India (Sanyal *et al.* 1975) and Sweden (Wadstrom *et al.* 1976, Ljungh *et al.* 1981). Other investigators

subsequently confirmed the role of this enterotoxin in the pathogenesis of *Aeromonas* (Boulanger *et al.* 1977, Dobrescu *el al.* 1978). Besides, aeromonads produce two types of hemolysins (α and β) that may play important role in hemorrhage and necrosis in *Aeromonas*-associated wound infection (Ljungh *et al.* 1982a and 1982b, Thelestam and Ljungh 1982). β-hemolysin of *Aeromonas* spp. can induce fluid accumulation in ligated rabbit ileal loop (RIL) (Stelma *et al.* 1986). Hemolysin-free purified *Aeromonas* enterotoxin could induce rounding of Y-1 mouse adrenal cells (without death), stimulated cyclic adenosin monophosphate synthesis and steroid secretion in Y-1 cells and fluid accumulation in RIL (Ljungh *et al.* 1981, Ljungh *et al.* 1982c). Possibly due to close taxonomic relationship between *Aeromonas* spp. and *Vibrio cholerae,* enterotoxin of *Aeromonas* spp. might have structural similarities with that of cholera toxin. A number of investigators proved it through immunological cross-reaction of *Aeromonas* enterotoxin with cholera toxin (James *et al.* 1982, Honda *et al.* 1985, Schultz and McCardell 1988, Chopra *et al.* 1996). However, Ljungh and Wadstrom (1982d) failed to show immunological cross-reaction of *Aeromonas* enterotoxin with cholera toxin. This discrepancy might be due to differences among isolates from different geographic locations.

Production of different virulence associated factors of *Aeromonas* spp. was studied in various bacteriological culture media with and without supplement (Karunakaran and Devi 1994, Tsai *et al.* 1997), meat extract (Majeed *et al.* 1991), body fluid (Ala Aldeen and Barer 1989, Wilkox *et al.* 1994) and synthetic medium (Riddle *et al.* 1981). Production of virulence factors such as enterotoxin

may be influenced by various environmental factors such as temperature, media composition, pH, salt concentration and dissolved oxygen (Tsai *et al.* 1997). *Aeromonas* can produce exotoxin due to prolonged incubation in beef-extract at lower temperature (Majeed *et al.* 1991).

Cytotoxic enterotoxin gene (*act*) of a diarrheal isolate *A. hydrophila* SSU has been cloned, sequenced and hyperexpressed (Chopra *et al.* 1993) to study the role of enterotoxin in the pathogenesis of this organism. Biological activities of this protein have been tested (Rose *et al* 1989a and 1989b). This study was undertaken to examine the effect of Ca^{2+}, Mg^{2+}, Fe^{3+} and NaCl on the production of cytotoxic enterotoxin (Act) of *A. sobria* isolated from the environment.

5.2. MATERIALS AND ME THODS

5.2.1. Bacterial strain: The strain *A. sobria* (strain no. 71), used in this experiment, was isolated from pond water. This strain possesses cytotoxic enterotoxin gene (*act)* and culture filtrate of this strain showed cytotoxicity in Vero cell-line (Asao *et al.* 1984). The strain was stored at room temperature in T_1N_1 (1.0% trypticase, 1.0% NaCl, agar 7.0%, pH 7.2) soft agar.

5.2.2. Media and culture conditions: Various liquid culture media were evaluated in this study. These included: brain heart infusion broth (BHIB, DIFCO, USA), trypticase soy broth with yeast extract (TSB, DIFCO USA), casamino acid yeast extract (CYE) and Richardson's medium (Richardson 1989)

5.2.3. Preparation of overnight culture: Stock culture from T_1N_1 soft agar was aseptically sub-cultured onto gelatin agar plate and incubated overnight at 37°C.

Following incubation, one well-isolated colony was inoculated into 10 ml BHIB and incubated overnight at 37°C in a shaking (120 oscillation per min) water bath (Water bath shaker, MM-10, Ogawa Seiki Co., LTD., Tokyo, Japan).

5.2.4. Divalent cations experiment: Five hundred millimole (mom) stock solution of calcium chloride ($CaCl_2$) and magnesium sulphate ($MgSO_4.H_2O$) was prepared and sterilized by filtration (0.22 μm pore; Millipore Corporation, USA). $CaCl_2$ and $MgS0_4$ (H_2O) were added to freshly prepared and sterilized BHIB to a final concentration of 1 mM, and 10 mM, respectively. These media were incubated overnight at 37°C to ensure sterility. 0.5 ml overnight broth culture of *A. sobria* was added to 10 ml sterilized media and incubated overnight at 37°C in a shaking water bath (120 oscillation per min). Following incubation, bacterial concentration was determined on a nutrient agar plate (DIFCO) following drop plate technique (Hoben and Somasegaran 1982). To prepare the supernatant, overnight culture BHIB (5.0 ml) was centrifuged at 10,000 rpm using Sorvall centrifuge (RC5C, Sorvall Instruments, DUPONT, USA) at 4°C. The supernatant was collected in another pre-sterilized glass vial and further sterilized by filtration (0.22 μm pore size; Millipore Corporation, USA). This experiment was repeated twice.

5.2.5. Iron experiment: Five hundred mM stock solution of ferric chloride ($FeCl_3$) was prepared and sterilized by filtration as above. $FeCl_3$ solution was added to freshly prepared and sterilized 10 ml BHIB to a final concentration of 200 and 2000 μM in separate conical flasks. These media were pre-incubated overnight at 37°C to ensure sterility. Contaminated media were discarded. 0.5 ml

overnight broth culture of *A. sobria* was added to 10 ml BHIB and incubated overnight at 37°C in a shaking water bath (120 oscillation per min). Following incubation, bacterial concentration was determined on a nutrient agar plate (DIFCO, USA) following drop plate technique (Hoben and Somasegaran 1982). To prepare the supernatant, overnight BHIB culture (5.0 ml) was centrifuged at 10,000 rpm using Sorvall centrifuge at 4°C. The supernatant was collected in another pre-sterilized glass vial and further sterilized by filtration as mentioned above. This experiment was repeated twice.

5.2.6. Sodium chloride experiment: BHIB was prepared according to the instructions of the manufacturer. To each 10 ml aliquot of BHIB, NaCl was added to a final concentration of 0.5%, 1.0%, 1.5% and 2.0% in different flasks prior to sterilization. Each of the flasks was inoculated with 0.5 ml overnight broth culture and incubated overnight at 37°C in a shaking water bath (120 oscillation per min). Following incubation, bacterial density was determined on a nutrient agar plate (DIFCO) following drop plate technique (Hoben and Somasegaran 1982). To prepare the supernatant, overnight culture was centrifuged at 10,000 rpm using Sorval centrifuge at 4°C. The supernatant was collected in another pre-sterilized glass vial and further sterilized by filtration as mentioned above. This experiment was repeated twice.

5.2.7. Vero cell assay: Cytotoxic effect of the culture filtrate containing crude cytotoxic enterotoxin was tested in Vero cell-line (African green monkey kidney cells) (Asao *et al.* 1984). Vero cell-line was grown in tissue culture medium 199 supplemented with Eagles salts and L-glutamate (2.3 mM), heat inactivated fetal

bovine serum (10%) (GIBCO BRL, USA), penicillin (100000 U/L), streptomycin ((100 mg/L) and gentamycin (50 mg/L). Two hundred microliters Vero cell suspension in tissue culture medium 199 ($2x10^3$/ 200 µl) was added in each well of microtiter plate (96 well, flat bottom, Falcon, USA) and incubated at 37°C in a humidified CO_2 incubator for three hours to allow adhesion. Following incubation, 200 µl diluted culture filtrate (serial two-fold dilution of crude toxin in PBS) was added into each well of the microtiter plate. Plate containing Vero cell and toxin were incubated at 37°C in humidified CO_2 incubator. Change of the cell morphology (cell death or rounding) was studied using an inverted phase contrast microscope after 18-24 hours of incubation. Wells containing 50% affected cells were considered positive.

5.3. RESULTS

5.3.1. Media for production of cytotoxic enterotoxin (Act): Of the four liquid media tested, the highest titer of Act was produced in BHIB. Therefore, subsequent experiments were conducted using BHIB medium.

5.3.2. Effect of calcium and magnesium on production of Act: The effect of calcium on the production of Act was studied in BHIB supplemented with $CaCl_2$. Compared to BHIB without supplemented, 2-fold rise of Act titer was observed when BHIB was supplemented with 1 mM $CaCl_2$. At this concentration of $CaCl_2$, density of *Aeromonas* decreased by one log compared to BHIB without

Table-5.1. Effect of different supplements of BHIB on production of Act and bacterial growth of an *act* gene positive *A. sobria* isolate

Treatments	Concentration	Cytotoxin titre	Bacterial cell concentration (No./ml)
None	-----	120	2.2×10^{10}
Calcium chloride	1.0 mM	240	7.5×10^{9}
	10.0 mM	120	2.2×10^{10}
Magnesium sulphate	1.0 mM	120	3.39×10^{10}
	10.0 mM	320	3.6×10^{10}
Ferric chloride	200 µM	160	6.4×10^{10}
	2000 µM	160	1.7×10^{10}
Sodium chloride	0.5%	160	1.7×10^{10}
	1.0%	120	2.1×10^{10}
	1.5%	120	6.1×10^{10}
	2.0%	80	4.5×10^{9}

supplement. When BHIB was supplemented with 10 mM $CaCl_2$, production of Act decreased but the bacterial concentration was comparable with that of BHIB without supplement (Table-5.1).

BHIB was supplemented with magnesium sulphate to study the effect of Mg^{2+} on the production of Act. Supplementation of BHIB with 10 mM $MgSO_4$ induced production of 2.7-fold rise of Act titer compared to plain BHIB. However, addition of 1 mM $MgSO_4$ did not increase production of Act compared to plain BHIB. Bacterial concentrations in BHIB supplemented with above two concentrations of $MgSO_4$ were comparable with that of BHIB without supplement. These results suggest that divalent cations, such as $Ca^{2+}$and Mg^{2+} may be required to supplement BHIB for the production of higher titer of Act (Table-5.1).

5.3.3. Effect of iron supplementation on production of Act: BHIB was supplemented with $FeCl_3$ to study the effect of iron on the production of Act. Supplementation of BHIB with both 200 and 2000 μM $FeCl_3$ indicated a 1.3 fold rise of Act production compared to BHIB without supplement. Bacterial growth in BHIB supplemented with the above two concentrations of $FeCl_3$ was comparable to that of BHIB without supplement (Table-5.1).

5.3.4. Effect of sodium chloride supplementation on production of Act: BHIB was supplemented with different concentrations of NaCl to study its effect on the production of Act. Compared to plain BHIB, Act titer slightly increased due to

supplementation of BHIB with 0.5% NaCl. Supplementation of BHIB with 1.0 and 1.5% NaCl induced production of Act comparable with that of BHIB without supplement. Bacterial growth due to supplementation of BHIB with 0.5, 1.0 and 1.5% NaCl was comparable with that of plain BHIB. Both bacterial growth and Act production were inhibited when BHIB was supplemented with 2.0% NaCl (Table 5.1).

5.4. DISCUSSION

Cytotoxic enterotoxin (*act*) gene has been cloned and sequenced from a diarrheal isolate *A. hydrophila* SSU (Chopra *et al.* 1993). The cytotoxic enterotoxin (Act) is a single polypeptide of 52 kDa (Rose *et al.* 1989a). Cytotoxicity is one of the biological activities of Act (Rose *et al.* 1989b). This toxin is secreted as an inactive precursor and subsequently converted into an active form by proteolytic activities (Chopra *et al.*1993). This protein makes pores on the membranes of erythrocyte (Ferguson *et al.* 1997). Hemolytic activity of Act is affected when pre-incubated with cholesterol. In this study, *A. sobria* was grown in BHIB supplemented with divalent cations ($Ca^{2+}$and Mg^{2+}), iron and different concentrations of sodium chloride to study their effect on the production of Act, which was tested in Vero cell-line.

In this study, four media {brain heart infusion broth (BHIB, DIFCO, USA), trypticase soy broth with yeast extract (TSB, DIFCO, USA), casamino acid yeast extract (CYE) and Richardson's medium} were evaluated to determine

optimum medium and condition for production of Act. In this study, the highest titer of cytotoxin was produced in BHIB. This result is consistent with the finding of Tsai *et al.* (1997). It is possible that some components of these media enhance production and subsequent transportation of Act outside the cell wall. On the other hand, inability to produce significant quantities of Act in other media might be due to inhibition of toxin synthesis, or failure to transport significant quantities of toxin outside the cell wall.

Metallic ions are used as co-factors of different enzymes. Supplementation of culture medium with some of these ions stimulates bacterial growth and production of pathogenic, such as β-hemolysin of *A. caviae.* (Karunakaran and Devi 1994). In the present investigation, enhanced production of Act due to the supplementation of BHIB with Ca^{2+} and Mg^{2+} indicates a similar mechanism of toxin production in *A. sobria* having cytotoxic enterotoxin gene (*act*). This observation is also supported by experiment relating to regulation of *act* in the presence of Ca^{2+} (Sha *et al.* 2001).

Bacterial growth requires soluble iron as one of the essential nutrients required for many biological processes of the organism including electron transport chain and also as co-factor of certain enzymes (Neilands 1981). At iron limiting condition, *Aeromonas* spp. produces iron-chelating compound known as siderophores. It helps *Aeromonas* to solubulise iron and makes it bioactive. In this study, supplementation of BHIB with iron slightly increased production of

Act. This observation is inconsistent to β-hemolysin production by A. *caviae* (Karunakaran and Devi 1994).

Sodium chloride is one of the important components of a bacteriological culture medium. Addition of sodium chloride to the medium enhances the production of virulence factors, such as enterotoxin (Nishibuchi *et al.* 1988) and hemolysin (Tison and Kelly 1984, Rahim and Aziz 1996) for some members of *Vibrionaceae*. Excess amounts of sodium chloride may adversely affect bacterial growth. In the present study, Act production increased with addition of 0.5% sodium chloride in BHIB. With gradual increase in the concentration of sodium chloride in the medium, production of cytotoxin gradually decreased. This trend is consistent with the production of hemolysin by strains of *V. fluvialis* (Rahim and Aziz 1996) and production of cytotoxin and hemolysin of *A. hydrophila* (Tsai *et al.* 1997) in BHIB.

This study demonstrated that BHIB induced production of higher titer of Act compared to other media tested. The production of Act was enhanced when BHIB was supplemented with optimum concentrations of iron, calcium, magnesium and sodium chloride. Further study is needed to understand the mechanism of action of these chemicals in the pathogenesis of *A. sobria in vitro* and *in vivo*.

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CHAPTER - 6

DETECTION OF PILUS AND O:34 ANTIGEN AMONG CYTOTOXIC ENTEROTOXIN GENE-POSITIVE ISOLATES OF *AEROMONAS* SPP.

6. DETECTION OF PILUS AND O:34 ANTIGEN AMONG CYTOTOXIC ENTEROTOXIN GENE-POSITIVE ISOLATES OF *AEROMONAS* SPP.

6.1. INTRODUCTION

The generally accepted mechanisms of exerting enteropathogenicity by enteric pathogens are adhesion to intestinal epithelium, colonization and secretion of toxins or invasion into enterocytes. Intestinal colonization by *Aeromonas* is mediated by non-structural cell wall components known as lectin (Ascensio *et al.* 1991). Different types of lectins of *Aeromonas* spp. have been reported (Roberts and Steigbigel 1977, Ascensio *et al.* 1991). Pili are structural cell-wall components and are also associated with colonization. *Aeromonas* pili are called type IV pili because of its structural similarities with type IV pili of *Neisseria and Pseudomonas* (Barnett *et al.* 1997). Human fecal isolates can produce two distinct families of type IV pili (Barnett *et al.* 1997). Human diarrheal isolates of *A. veronii* bv. *sobria* and *A. caviae* can produce bundle-forming pili (Bfp) when grown under *in vitro* condition (Kirov and Sanderson 1996, Kirov *et al.* 1998). Bfp pili are important colonization factors (Kirov *et al.* 1999) and have also been isolated from *A. hydrophila.* Some isolates of *A. hydrophila* can produce a profuse amount of type I pili (Honma and Nakasone 1990). N-terminal sequence of Bfp of *A. sobria* shows homology with mannose-sensitive hemagglutination pili of *V. cholerae.* Bfp has been purified from all species of *Aeromonas* isolated from diarrheal stool but these pili have not been characterized at molecular level (Kirov *et al.* 1999). TAP is another class IV type pilus of *A. sobria strain* TAP13 (Iwanaga and Hokama 1992). This pilus was initially isolated from *A. hydrophila*

(strain Ah65)(Pape *et al.* 1996). TAP pilus differs from Bfp with respect to molecular weight and N-terminal amino acid sequence. However, the N-terminal amino acid sequence of TAP pilus shows homology with type IV pilus of *Pseudomonas aeruginosa* and *Neisseria* spp. (Barnett *et al.* 1997). However, there is no report of serotype-specific pilus of *Aeromonas.*

Biochemically similar isolates of *Aeromonas* spp. could be easily differentiated by their antigenic structure of the cell wall. Based on the cell-wall antigenic structure, various serotyping schemes have been proposed by different groups of scientists (Sakazaki and Shimada 1984, Cheasty *et al.* 1988, Misra *et al.* 1989, Merino and Tomas 1988). Of various serotypes, O:34 has been increasingly isolated from cases of septicemia and wound infections in humans (Kokka *et al.* 1991, Janda *et al.* 1994). This particular serotype of *Aeromonas* was incriminated as one of the etiological agents associated with mortality due a severe fish disease (Lallier *et al.* 1984, Merino *et al.* 1989). Therefore, *Aeromonas* spp. belonging to the serotype O:34 are considered pathogenic for fish and human beings.

In Bangladesh, strains of *Aeromonas* have been isolated from various sources, such as infected fish (Rahim *et al.* 1984, 1985), prawn (Rahim and Aziz 1994) and some biotic components of a pond ecosystem (Islam *et al.* 1992). Some isolates showed hemolytic activity and enterotoxicity (Rahim *et al.* 1984, Rahim and Aziz 1994) but isolates from Bangladesh have not been tested for the presence of pili and O:34 antigen. In the present study, cytotoxic enterotoxin gene-positive isolates were tested for these two parameters.

6.2. MATERIALS AND METHODS

6.2. Bacterial isolates: Thirty-six cytotoxic enterotoxin (*act*) gene-positive isolates of *Aeromonas* spp. were used in this study. These strains were isolated from duckweed (*Lemna minor*), water and fish samples during August 1994 through July 1995. The *act* gene was detected by using hybridization of colony blots with 0.6 kb restriction fragment of plasmid pXHC95 (Xu et *al.* 1998).

6.2.2. Preparation of O-antigen: Bacterial type strain *A. hydrophila* Aer 223 was obtainted from Dr. M. Janda, USA. The strain was subcultured on MacConkey's agar plate and incubated at 37°C overnight. Following incubation, one well-isolated typical aeromonad colony was inoculated on tryptic soy agar slant containing glucose (0.1%) and incubated overnight at room temperature. Following incubation, the culture was resuspended in 0.85% NaCl solution from the slant and boiled for 1.5 hours. Boiled cells were washed three times in 0.85% NaCl solution and finally resuspended in 0.85% NaCl with a turbidity of 1 at OD_{540}.

6.2.3. Rabbit immunization: New Zealand white rabbit weighing 1.2 to 1.9 kg were used for raising sera corresponding to O:34 antigen of type strain *A. hydrophila* Aer 223 following the procedure of Janda *et al.* (1987). Two rabbits were immunized. Before initiating the immunization schedule, 5 ml blood was collected from each rabbits to obtain pre-immune sera to use as negative control for agglutination test.

Rabbits were immunized through the ear vein with an increasing volume of antigen twice a week for three weeks, for example, first week 0.25 and 0.5 ml, second week 1.0 and 2.0 ml and third week 3.0 ml. During the course of immunization, concentrations of cells were always maintained the same as mentioned above. On fourth week, serum from 5.0 ml of blood was collected from both the immunized rabbits and the antibody titer was measured corresponding to the type strain used for immunizing the rabbits. In case of low titer, the rabbit was boostered once and immune serum was collected on the following week. Antibody titer corresponding to the type strain was measured by the tube dilution method (Albert *et al.* 1995).

6.2.4. Detection of O:34 antigen: Isolates harboring *act* gene were sub-cultured on Luria-Bartani (L-B) agar slant and incubated at 37°C overnight. Following incubation, culture was resuspended in 0.85% NaCl solution and boiled for 1.5 hours samples. Boiled cells were washed three times in 0.85% NaCl solution. Washed cells were emulsified with immune sera on a clean glass slide for checking the presence of O:34 antigen on the cell wall (Kokka and Janda 1990).

6.2.5. Electron microscopy: *Aeromonas* isolates were retrieved from the stock culture on L-B agar plate at 37°C. One isolated typical aeromonad colony was inoculated into 5.0 ml L-B broth and incubated overnight aerobically at 37°C without shaking. Following incubation, culture was placed on carbon grid, stained with 2.0% uranyl acetate and examined under 420T transmission electron microscope for pili and flagella.

6.3. RESULTS AND DISCUSSION

Atkinson *et al.* (1987) reported an outer membrane protein of 43 kDa. colonization factor antigen of *Aeromonas*. This protein was designated as A6-HAG. It agglutinated erythrocytes of different species. But its role in the colonization of intestinal wall was not proved. On the other hand, Hokama *et al.* (1990) reported wavy-flexible pili from *A. hydrophila* Ae6 and experimentally proved its ability to colonize the human and rabbit intestine. In this study, 27 *act*-gene positive isolates of *Aeromonas* spp. were included. Of them, 8 isolates were *A. hydrophila* and 19 were *A. sobria*. These strains were isolated water (n=13), duckweed (n=10), gills (n=2) and intestinal contents (n=2) of fish. Pili were detected in 13 isolates. Of them, 11 isolates were *A. sobria* and 2 were *A. hydrophila*. These were isolated from water (n=5), duckweed (n=4), gills (n=2) and intestinal contents (n=2) of fish (Table-6.1).

Generally, *Aeromonas* spp. produce various types of pili. Some strains produce long, wavy and flexible types of pili (Hokama and Iwanaga 1992, Iwanaga and Hokama 1992), while others produce both flexible and rigid type (Honma and Nakasone 1990, Hokama *et al.* 1990). Rigid pili of *A. hydrophila* Ae6 are associated with auto-agglutination (Honma and Nakasone 1990) but wavy–flexible pili of *A. hydrophila* Ae6 play a role in adhesion to intestinal wall. Strains tested in this study produced wavy flexible type of pili (Fig. 6.1). Further study is needed to understand the role of these pili in pathogenesis of *Aeromonas*.

Fig. 6.1. Electron micrograph showing pili (hollow arrow), flagella (solid arrow) and aeromonad cell (hollow trangle) (69, 000 X)

Aeromonas strains that belong to O:34 serogroup are highly virulent in animal models some strains of this serotyppe consistently produce structural and enzymatic properties associated with human infections (Khashe *et al.* 1996). In this study, O:34 antigen was detected among 11 isolates. Of them, 9 isolates were *A. sobria* and 2 were *A. hydrophila*. O:34 antigen-positive strains were isolated from water (n=3), duckweed (n=6), gills (n=1) and intestinal contents (n=1) of fish (Table-1). Detection of O:34 antigen has been reported among the isolates from human, animal and environmental sources (Khashe *et al.* 1996). However, in this study, all the strains were isolated from the aquatic environment and the majority of the *A. sobria* isolates harbored O:34 antigen, whereas Khashe *et al.* (1996) detected O:34 antigen mostly in *A. hydrophila* strains isolated from different sources, including human, animal and environment. This discrepancy may be due to geographical and environmental difference of the isolates. In this study, pili and O:34 antigen were detected more among *A. sobria* isolates than among *A. hydrophila* isolates. These results suggest that some *act* gene- positive isolates of *A. sobria* and *A. hydrophila* possess organs for adhering to intestinal epithelium for initiating enteric infection. Further experiments in tissue culture and animal model are needed to confirm the pathogenic behavior of these isolates having pili and *act* gene. However, there is no relationship of pilus and O:34 serotype with the presence of *act* gene in *Aeromonas* spp.

Table-6.1. Detection of pili, flagella and O:34 antigen in *act* gene-positive isolate of *Aeromonas* spp.

Isolate no.	Pili	Flagella	O: 34 antigen	Sources	Species
25	+	+	+	W	*A. sobria*
26	-	+	+	W	*A. sobria*
33	+	+	+	Fg	*A. sobria*
43	+	+	-	Dw	*A. sobria*
48	-	+	-	Dw	*A. sobria*
51	-	+	+	Dw	*A. sobria*
52	-	+	+	Dw	*A. sobria*
55	-	+	+	Dw	*A. sobria*
58	+	+	-	W	*A. hydrophila*
59	+	+	+	FI	*A. sobria*
64	+	+	-	FG	*A. sobria*
71	+	+	-	W	*A. sobria*
75	-	+	-	W	*A. sobria*
77	+	+	-	W	*A. sobria*
79a	-	+	-	W	*A. hydrophila*
85	+	+	+	Dw	*A. hydrophila*
91	-	+	+	Dw	*A. sobria*
97	+	+	+	Dw	*A. sobria*
107	+	+	+	Dw	*A. sobria*
137	-	+	-	W	*A. hydrophila*
139	-	+	-	W	*A. hydrophila*
155	-	+	-	W	*A. hydrophila*
167	-	+	-	W	*A. hydrophila*
176	-	+	+	W	*A. hydrophila*
177	+	+	-	Dw	*A. sobria*
187	+	+	-	W	*A. sobria*

W=water, DW= duckweed, FG= Fish-gills, FI= fish-intestinal contents

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CHAPTER -7

CONCLUDING REMARKS

7. CONCLUDING REMARKS

Aeromonas spp. are autochthonous flora of aquatic environment. In Bangladesh this bacterium was isolated from different aquatic ecosystems including ponds, river, lake, aquatic plants, phyto-and zooplankton, ulcerated fish, prawns as well as from feces of diarrheal and non-diarrheal patients. *Aeromonas* isolates from these sources of Bangladesh showed toxigenic potential when tested in animal models and cell-line. Some of the isolates were resistant to antibiotics, such as ampicillin, erythromycin, tetracycline etc. Therefore, presence of *Aeromonas* in the aquatic environment indicates public health problem. However, there are some information-gap on the impact of physicochemical parameters of water on *Aeromonas* density, density of *Aeromonas* phage and pathogenic potential of environmental *Aeromonas* spp. harboring cytotoxic enterotoxin gene. Moreover, presence of pilus, drug resistance pattern and pathogenic potential of cytotoxic enterotoxin gene-positive isolates of *Aeromonas* spp. have not been addressed earlier.

Aeromonas was detected in all types of samples studied including water, aquatic plant and fish through out the year. Of the various sampling sites, the highest density of *Aeromonas* was detected in untreated sewage water. There was no apparent seasonality of *Aeromonas* isolated from different kinds of sample. However, *Aeromonas* density in water showed two apparent peaks one in January-February and another in May. Further studies are suggested to confirm seasonality and incidence of *Aeromonas* spp.

Correlation of physicochemical parameters of water with the incidence and density of *Aeromonas* is an important finding of this study. *Aeromonas* density in gills and intestinal contents of fish correlated with pH and conductivity in non-wastewater

area ($P<0.001$) and only with temperature in both wastewater ($P<0.01$) and non-wastewater areas ($P<0.001$). *Aeromonas* density of water correlated well with conductivity ($P<0.001$) only in wastewater area. However, uniform correlation of *Aeromonas* density with all the physicochemical parameters was not observed in both the sampling sites. It is possibly due to analysis of few samples for a period of one year. Similar studies should be continued to determine seasonality.

Hemolysin is one of the pathogenic factors of *Aeromonas*. Hemolytic activity correlates with enterotoxicity. Both α and β hemolytic activities were detected in all the three species of *Aeromonas*, such as *A. hydrophila*, *A. sobria* and *A. caviae*. Published literature suggests that β-hemolysin is more toxic than α–hemolysin. It is remarkable to mention here that for the first time in Bangladesh, β-hemolytic activity was detected in isolates of *A. caviae*. Detection of β-hemolysin in isolates of *A. caviae* strengthens the argument that strains of this species are equally pathogenic like other *Aeromonas* species isolated from the environment of Bangladesh.

Duckweed (DW) can be grown m wastewater for treatment of polluted water and the same DW can be used as fish-feed without adversely affecting pisciculture. Sewage grown DW is also safe for persons handling DW and fish. These observations suggest that, duckweeds can be used as safe biological agent for wastewater treatment. The present findings suggests that simultaneously the same DW can also be used as fish-feed.

A. hydrophila phages are distributed in sewage-contaminated and uncontaminated pond samples of Bangladesh. This is for the first time *Aeromonas* phages have been isolated from Bnagladesh. Concentrations of *Aeromonas* phages are more in uncontaminated than sewage-contaminated samples. These phages are

specific for *Aeromonas*. It is essential to extend this work to constitute phage-typing scheme for rapid identification and typing of *A. hydrophila* strains.

Cytotoxic enterotoxin is one of the virulence factors in the pathogenesis of *Aeromonas* spp. Gene for cytotoxic enterotoxin (*act*) was detected in half of the environmental isolates of *Aeromonas* spp. examined in this study. However, all the *act* gene-positive isolates could not induce enterotoxicity uniformly in animal models and cell-line tested. It is possibly due to the fact that strains failed to induce toxicity might have truncated genes, which is unable to produce biologically active toxin. This mutated toxin fail to exert toxicity in standard animal model and cell-line. To confirm this hypothesis, gene for probe-positive isolates should be sequenced and compared with wild-type gene coding for *act*. The other possibility is, cytotoxic enterotoxin produced in the nucleus is inactivated subsequently when it is excreted outside the cell. Further work is needed to confirm this hypothesis.

Diarrhea is a self-limiting disease. Thus, antibiotic is not required for the treatment of this disease. However, in order to shorten the duration of diarrhea, very often antibiotic is prescribed by the physicians for the comfort of the patients. Therefore, antibiotic susceptibility testing data is required for prescribing antibiotics for the treatment of *Aeromonas*-associated diarrhea. Testing of antibiotic sensitivity patterns of both toxin gene-positive and gene-negative isolates showed variable sensitivity patterns with respect to commonly used antibiotics. It is alarming to note that these isolates (toxin gene-positive and gene-negative) were uniformly sensitive to chloramphenicol, ciprofloxacin and furazolidon. It indicates that enteric pathogens have acquired resistance against commonly used antibiotics as a result of abusing

them in any kind of mild infection. Therefore, national guideline should be formulated for proper use and dispensation of antibiotics in the community.

Gene for cytotoxic enterotoxin (*act*) was detected in strains of *Aeromonas* spp. But the culture media and growth condition for the better production of this toxin should be studied further. We have found that brain heart infusion broth (BHIB) is the ideal medium for the better production of Act. Optimum concentration of different supplements (iron, calcium, magnesium and sodium chloride) has been determined for better production of Act in BHIB. Therefore, it is suggested to use BHIB with the supplements for the better production of Act. These conditions could be used for the production, purification and immuno-biological characterization of Act.

Pilus is one of the pathogenic factors of *Aeromonas* spp. This is for the first time we have detected it in toxin gene-positive isolates of *Aeromonas* spp. But due to limitation of time and facilities, pothegenic properties of these pili could not be studied. Therefore, further research should be addressed to purify pilus protein for preparing anti-pilus antibody to conduct adhesion-inhibition experiment of piliated *Aeromonas* spp. in cell-line and intestinal tissue of different laboratory animals. Further studies are recommended for the characterization of pathogenic behavior of these pilus.

CHAPTER - 8

Appendix

Table-8.1. *Aeromonas* density in duckweed samples of wastewater and non-wastewater areas.

Months	Non-wastewater				Wastewater			
	B1	B2	CP3	CP4	L6	L7	P8	P10
Aug 94	$4.0x10^6$	$2.0x10^7$	$1.9x10^7$	$4.6x10^7$	$6.0x10^6$	$1.0x10^6$	$2.8x10^8$	$4.0x10^6$
Sept 94	$4.0x10^8$	$2.0x10^7$	$1.8x10^7$	$8.0x10^8$	$1.6x10^9$	$1.4x10^7$	$9.0x10^7$	$2.4x10^7$
Oct 94	$8.0x10^6$	$1.8x10^7$	$4.0x10^5$	$4.0x10^5$	$4.0x10^5$	$1.4x10^5$	$4.0x10^6$	$4.0x10^4$
Nov 94	$4.6x10^7$	$2.0x10^1$	$1.0x10^2$	$2.6x10^2$	$6.0x10^5$	$6.0x10^5$	$6.0x10^5$	$4.0x10^5$
Dec 94	$4.0x10^5$	$2.0x10^4$	$4.0x10^5$	$8.0x10^5$	$1.4x10^6$	$4.0x10^5$	$3.6x10^6$	$2.0x10^6$
Jan 95	$1.0x10^4$	$6.0x10^5$	$3.8x10^6$	$1.4x10^5$	$1.8x10^5$	$1.0x10^5$	$6.0x10^3$	$6.4x10^6$
Feb 95	$6.0x10^4$	$1.4x10^5$	$5.2x10^5$	$2.4x10^5$	$1.8x10^5$	$1.4x10^5$	$1.3x10^5$	$7.4x10^5$
Mar 95	$1.8x10^5$	$2.6x10^5$	$1.4x10^6$	$1.1x10^6$	$1.2x10^5$	$3.8x10^5$	$2.0x10^5$	$7.0x10^6$
Apr 95	$3.2x10^5$	$2.4x10^5$	$2.4x10^4$	$4.0x10^4$	$2.6x10^5$	$1.4x10^5$	$1.2x10^6$	$1.0x10^6$
May 95	$1.8x10^5$	$4.0x10^4$	$1.6x10^5$	$8.0x10^4$	$4.0x10^4$	$4.0x10^4$	$4.0x10^5$	$4.0x10^5$
Jun 95	$2.0x10^5$	$6.0x10^4$	$8.0x10^4$	$1.8x10^5$	$1.0x10^5$	N/A	$4.0x10^4$	$2.0x10^4$
Jul 95	$2.0x10^4$	$2.0x10^4$	$6.0x10^4$	$4.0x10^4$	$2.0x10^4$	$4.0x10^4$	$4.0x10^4$	$2.0x10^4$

B_1 = Duckweed growing bed 1

B_2 = Duckweed growing bed 2

CP_3 = Fish growing pond 1

CP_4 = Fish growing pond 2

Table-8.2. *Aeromonas* density in water samples of wastewater and non- wastewater areas.

Months	Non-wastewater				Wastewater				
	B1	B2	CP3	CP4	L5	L6	L7	P8	P10
Aug 94	$2.0x10^1$	$4.1x10^2$	$7.0x10^1$	$8.0x10^2$	$3.0x10^3$	$1.4x10^2$	$2.5x10^2$	$3.0x10^1$	$4.4x10^2$
Sept.94	$7.0x10^1$	$3.0x10^1$	$2.0x10^1$	$3.0x10^1$	$2.0x10^3$	$2.0x10^1$	$7.0x10^1$	$4.0x10^1$	$7.0x10^1$
Oct 94	$5.0x10^1$	$5.0x10^1$	$4.0x10^1$	$4.0x10^1$	$2.0x10^3$	$2.0x10^1$	$5.0x10^1$	$3.0x10^1$	$2.0x10^1$
Nov 94	$2.1x10^2$	$1.3x10^2$	$4.0x10^1$	$2.2x10^2$	$2.0x10^3$	$2.9x10^2$	$1.3x10^2$	$2.0x10^1$	$8.0x10^1$
Dec 94	$1.0x10^2$	$2.4x10^2$	$2.5x10^2$	$9.0x10^1$	$3.0x10^3$	$1.8x10^2$	$9.0x10^1$	$3.0x10^1$	$1.1x10^2$
Jan 95	$3.2x10^2$	$2.8x10^2$	$6.2x10^2$	$2.2x10^2$	$1.0x10^3$	$109x10^2$	$7.0x10^1$	$2.3x10^2$	$9.4x10^2$
Feb 95	$2.5x10^2$	$1.8x10^2$	$7.1x10^2$	$3.3x10^2$	$1.5x10^4$	$1.2x10^2$	$3.0x10^1$	$5.3x10^2$	$6.6x10^2$
Mar 95	$1.8x10^2$	$9.0x10^1$	$3.0x10^1$	$8.0x10^1$	$4.0x10^3$	$6.0x10^1$	$3.7x10^1$	$1.2x10^2$	$4.1x10^2$
Apr 95	$1.2x10^2$	$1.2x10^2$	$1.0x10^1$	$1.3x10^2$	$6.0x10^3$	$3.0x10^1$	$5.0x10^1$	$3.0x10^1$	$1.8x10^2$
May 95	$1.7x10^2$	$2.1x10^2$	$6.8x10^2$	$2.0x10^2$	$8.0x10^3$	$9.0x10^1$	$7.0x10^1$	$1.1x10^2$	$2.2x10^2$
Jun 95	$3.0x10^1$	$9.0x10^1$	$7.0x10^1$	$7.0x10^1$	$2.0x10^3$	$1.0x10^2$	$7.0x10^1$	$1.2x10^2$	$1.3x10^2$
Jul 95	$1.7x10^2$	$1.0x10^2$	$2.4x10^2$	$1.3x10^2$	$5.0x10^3$	$1.6x10^2$	$7.0x10^1$	$7.0x10^1$	$1.1x10^2$

B_1 = Duckweed growing bed 1

B_2 = Duckweed growing bed 2

CP_3 = Fish growing pond 1

CP_4 = Fish growing pond 2

Table-8.3. *Aeromonas* density in fish-gills of wastewater and non- wastewater areas.

Months	Non-wastewater		Wastewater	
	CP3	CP4	P8	P10
Aug 94	$4.0x10^8$	$4.0x10^8$	$1.2x10^7$	$6.0x10^6$
Sep 94	$2.2x10^7$	$2.6x10^8$	$3.0x10^7$	$8.0x10^6$
Oct 94	$1.0x10^5$	$6.0x10^5$	$6.0x10^5$	$4.0x10^5$
Nov 94	$2.0x10^4$	$6.0x10^5$	$8.0x10^5$	$8.0x10^5$
Dec 94	$8.0x10^4$	$1.2x10^5$	$6.0x10^4$	$4.0x10^4$
Jan 95	$1.2x10^5$	$8.8x10^4$	$1.0x10^4$	$4.0x10^4$
Feb 95	$1.0x10^5$	$6.0x10^4$	$3.4x10^5$	$1.8x10^5$
Ma 95	$2.0x10^3$	$1.4x10^5$	$2.0x10^4$	$3.8x10^5$
Apr 95	$6.0x10^4$	$6.0x10^4$	$8.0x10^4$	$8.0x10^5$
May 95	$4.0x10^4$	$8.0x10^4$	$8.0x10^4$	$6.0x10^5$
Jun 95	$6.0x10^3$	$2.6x10^4$	$1.6x10^4$	$1.2x10^4$
Jul 95	$4.0x10^3$	$1.4x10^4$	$1.4x10^4$	$4.0x10^3$

CP_3 = Fish growing pond 1
CP_4 = Fish growing pond 2
P_3 = Fish growing pond 2
P_{10} = Fish growing pond 3

Table-8.4. *Aeromonas* density in fish-intaestinal contents of wastewater and non- wastewater areas

Months	Non-wastewater		Wastewater	
	CP_3	CP_4	P_8	p_{10}
Aug 94	$2.8x10^{10}$	$2.2x10^{8}$	$1.1x10^{8}$	$6.0x10^{8}$
Sept 94	$1.2x10^{10}$	$1.8x10^{9}$	$1.8x10^{9}$	$2.2x10^{9}$
Oct 94	$1.0x10^{6}$	$6.0x10^{7}$	$6.0x10^{6}$	$4.0x10^{6}$
Nov 94	$4.4x10^{6}$	$5.4x10^{6}$	$6.0x10^{5}$	$6.0x10^{5}$
Dec 94	$3.6x10^{6}$	$1.8x10^{6}$	$3.2x10^{6}$	$7.4x10^{6}$
Jan 95	$1.7x10^{7}$	$6.6x10^{6}$	$1.7x10^{7}$	$1.5x10^{7}$
Feb 95	$3.1x10^{7}$	$8.0x10^{6}$	$6.4x10^{6}$	$3.0x10^{7}$
Mar 95	$2.1x10^{7}$	$3.0x10^{7}$	$5.4x10^{7}$	$2.8x10^{7}$
Apr 95	$8.0x10^{7}$	$4.4x10^{7}$	$1.2x10^{5}$	$7.6x10^{7}$
May 95	$8.0x10^{6}$	$1.1x10^{7}$	$1.8x10^{7}$	$4.6x10^{7}$
Jun 95	$2.0x10^{7}$	$6.0x10^{6}$	$1.0x10^{7}$	$1.0x10^{7}$
Jul 95	$1.2x10^{6}$	$1.2x10^{7}$	$2.0x10^{6}$	$6.0x10^{6}$

CP_3 = Fish growing pond 1
CP_4 = Fish growing pond 2
P_3 = Fish growing pond 2
P_{10} = Fish growing pond 3

Table-8.5. pH of different sampling sites of wastewater area

Months	Locations				
	L_5	L_6	L_7	P_8	P_{10}
Aug 94	6.99	7.25	7.56	7.53	8.61
Sept 94	7.10	7.40	7.80	7.80	8.80
Oct 94	7.50	7.50	7.70	8.00	8.20
Nov 94	7.40	7.50	6.60	7.20	7.50
Dec 94	7.10	7.30	7.70	7.00	8.60
Jan 95	7.50	7.80	8.30	7.50	8.40
Feb 95	7.30	7.70	8.00	7.30	8.70
Mar 95	7.80	7.30	7.60	7.10	8.00
Apr 95	7.40	7.20	7.40	7.10	7.60
May 95	8.25	7.35	7.40	7.80	7.63
Jun 95	7.40	7.40	8.30	8.30	8.00
July 95	7.50	7.00	7.90	7.50	7.60

L_5 = Raw sewage

L_6 = Stabilization pond 1

L_7 = Stabilization pond 2

P_3 = Fish growing pond 2

P_{10} = Fish growing pond 3

Table-8.6. pH of different sampling sites of non-wastewater area

Months	Locations			
	B_1	B_2	CP_3	CP_4
Aug 94	6.92	7.00	8.30	7.96
Sept 94	7.30	7.20	7.80	8.30
Oct 94	7.40	7.30	8.20	7.80
Nov 94	7.30	7.30	8.30	8.70
Dec 94	6.90	6.90	7.60	8.40
Jan 95	7.10	7.40	7.60	8.30
Feb 95	7.30	7.40	7.80	8.30
Mar 95	6.80	6.80	8.10	7.60
Apr 95	7.00	7.00	7.80	8.20
May 95	7.18	7.20	7.60	7.50
Jun 95	7.00	7.20	7.80	7.70
Jul 95	6.70	7.00	7.30	7.40

B_1 = Duckweed growing bed 1

B_2 = Duckweed growing bed 2

CP_3 = Fish growing pond 1

CP_4 = Fish growing pond 2

Table-8.7. Temperature (°C) of different sampling sites of wastewater area

Months	L_5	L_6	L_7	P_8	P_{10}
Aug 94	33.00	33.00	34.00	34.00	34.00
Sep 94	32.00	33.00	34.00	35.00	35.00
Oct 94	32.00	32.00	34.00	34.00	34.00
Nov 94	34.00	30.00	32.00	32.00	34.00
Dec 94	26.00	22.00	25.00	26.00	27.00
Jan 95	21.00	15.00	19.00	20.00	21.00
Feb 95	21.00	18.00	21.00	22.00	22.00
Mar 95	26.00	26.00	26.00	27.00	27.00
Apr .95	31.00	28.00	30.00	32.00	34.00
May 95	31.00	30.00	31.00	32.00	32.50
Jun 95	30.00	32.00	32.00	32.00	32.00
Jul 95	30.00	30.00	30.00	30.00	31.00

L_5 = Untreated raw wastewater

L_6 = DW based wastewater treatment pond 1

L- = DW based wastewater treatment pond 2

P_8 = Fish growing pond 2

P_{10} = Fish growing pond 3

Table-8.8. Temperature (°C) of different sampling sites of non-wastewater area

Months	Locations			
	B_1	B_2	CP_3	CP_4
Aug 94	33.00	34.00	34.00	34.00
Sep 94	32.00	32.00	34.00	35.00
Oct 94	31.00	31.00	35.00	34.00
Nov 94	29.00	29.00	32.00	32.00
Dec 94	23.00	25.00	25.00	26.00
Jan 95	19.50	19.50	20.00	20.00
Feb 95	18.00	18.00	20.00	21.00
Mar 95	23.0	23.00	25.00	25.00
Apr 95	27.00	27.00	30.00	31.00
May 95	30.00	29.00	31.00	31.50
Jun 95	30.00	30.00	32.00	33.00
Jul 95	28.00	28.00	30.00	30.00

B_1 = Duckweed growing bed 1

B_2 = Duckweed growing bed 2

CP_3 = Fish growing pond 1

CP_4 = Fish growing pond 2

Table-8.9. Conductivity (mS/m*) of different sampling sites of wastewater area

Months	L_5	L_6	L_7	P_8	P_{10}
Aug 94	100.00	49.00	41.00	41.00	36.00
Sept 94	104.00	57.00	42.00	34.00	41.00
Oct 94	93.00	55.00	47.00	38.00	43.00
Nov 94	92.00	55.00	46.00	43.00	45.00
Dec 94	105.00	60.00	53.00	33.00	44.00
Jan 95	98.00	65.00	59.00	50.00	52.00
Feb 95	115.00	74.00	57.50	55.00	47.00
Mar 95	105.00	59.00	59.00	51.00	49.05
Apr 95	112.00	56.00	58.00	50.00	56.00
May 95	56.00	50.00	43.00	44.00	48.00
Jun 95	72.00	49.00	45.00	45.00	40.00
Jul 95	59.00	35.00	31.00	34.50	32.00

mS/m*= millisiemens/meter

L_5 = Raw sewage

L_6 = Stabilization pond 1

L- = Stabilization pond 2

P_9 = Fish growing pond 2

P_{10} = Fish growing pond 3

Table-8.10. Conductivity (mS/m*) of different sampling sites of non-wastewater area

Months	Locations			
	B_1	B_2	CP_3	CP_4
Aug 94	42.0	44.00	44.00	46.00
Sept 94	40.0	44.00	47.00	45.00
Oct 94	46.0	45.00	49.00	49.00
Nov 94	45.50	49.00	46.00	45.00
Dec 94	44.00	47.00	50.00	50.00
Jan 5	49.00	52.00	47.00	50.00
Feb 95	47.50	49.00	54.00	54.50
Mar 95	49.50	49.50	49.00	49.00
Aprl 95	46.00	46.00	44.00	46.50
May 95	40.00	40.00	43.9 0	43.00
Jun 95	52.00	46.00	40.50	41.00
July 95	26.00	22.00	36.00	32.00

mS m*= millisiemens/meter

B_1 = Duckweed growing bed 1

B_2 = Duckweed growing bed 2

CP_3 = Fish growing pond 1

CP_4 = Fish growing pond 2

Table-11. Pathogenic potential of the *act* probe positive isolates of *Aeromonas* spp.

Strain no.	Suckling mice assay with culture filtrate		Vero cell assay with culture filtrate		Hemolysin assay (aerobic)	Hemolysin assay (candle jar)	CAMP hemolysin assay	Rabbit ileal loop assay
	Unheated	Heated at 56°C	Unheated	Heated at 56 °C				
25	-	-	+	-	-	-	+	-
26	+	-	+	–	α	–	+	+
33	+	-	+	-	α	α	+	+
38	-	-	-	-	α	α	-	-
43	+	-	+	-	α	α	-	-
48	+	-	+	-	α	α	-	-
51	-	-	+	-	α	-	+	nd
52	+	-	+	-	α	α	+	-
55	-	-	+	–	α	nd	+	nd
58	-	-	+	-	α	nd	-	nd
64	-	-	+	-	α	nd	+	nd
71	+	-	+	-	β	β	-	-
75	-	-	+	-	α	α	-	-
77	+	-	+	-	α	α	-	-
79a	+	-	+	-	α	α	-	-
85	-	-	+	-	β	β	-	-
91	-	-	+	-	α	α	+	-
93	-	-	-	-	β	β	+	+
97	-	-	+	-	β	α	+	-
107	-	-	+	-	β	β	+	-
137	+	-	+	-	β	β	-	-
139	+	-	+	-	β	β	-	-
155	-	-	+	-	β	α	-	-
156	nd	nd	+	nd	α	nd	-	nd
164	nd	nd	nd	nd	α	nd	-	nd
165	-	-	-	-	β	α	+	-
167	-	-	+	-	nd	nd	-	-
173	-	-	-	-	α	α	-	-
175	-	-	-	-	α	α	+	-
176	-	-	+	-	β	β	+	-
177	-	-	+	-	β	β	-	-
187	-	-	-	-	β	α	-	-

Table-12. Pathogenic potential of the *act* probe negative isolates of *Aeromonas* spp.

Strain no.	Suckling mice assay	Vero cell assay	Hemolysin assay (aerobic)	CAMP-hemolysin assay
17	-	+	α	+
18	-	+	α	-
22	-	+	α	+
30	-	+	-	-
31	-	+	β	+
44	-	+	β	-
50	-	+	–	-
59	-	+	α	+
66	+	+	α	-
71a	-	+	α	-
72	+	+	-	-
74	-	-	-	-
76	-	+	-	-
77	-	+	α	-
79	-	+	β	-
80	-	+	β	+
83	-	+	α	-
94	-	+	α	-
99	-	+	α	+
104	-	+	α	-
108	-	+	α	-
111	-	-	-	-
113	-	-	-	-
119	-	+	-	-
124	-	+	-	+
126	-	+	α	-
128	-	+	α	-
129	-	+	α	-
131	-	nd	α	-
137	-	+	α	+
139	-	+	α	nd

Nd=not done